Ecological immunology of fungal infections in Drosophila

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Summary

Organisms face a constant risk of attack from parasites. While classic immunology has revealed numerous physiological and molecular mechanisms that underpin host immunity, the recently developed field of ecological immunology has attempted to understand the ecological and evolutionary causes that explain the diversity of such immune mechanisms. However, progress in the field has been hampered by the complex relationship between immunity and fitness as well as the methodological limitations of our experiments. There is an urgent need for eco-immunological studies that combine life history theory with experimentally tractable but ecologically realistic host and pathogen models. In this thesis, I tackle three novel aspects of host defence against parasites in an established model for insect immunity, the fruit fly *Drosophila melanogaster*, with the entomopathogenic fungus *Metarhizium robertsii*, one of the most successful natural insect pathogens. In particular, I show in Chapter 2 that an immune and stress response gene, *Turandot M*, provides specific immunity against sexually transmitted fungal infections; but, this protective effect comes at a cost to life history in the absence of infection. In Chapter 3, I show that when exposed to the fungal pathogen, the fruit fly alters its temperature preference by seeking out cooler temperatures, which results in a dramatic shift in its life history strategy while simultaneously enhancing antifungal resistance, though not tolerance. Finally, I demonstrate in Chapter 4 that exposure to fungal parasites induces fitness-associated maternal effects on offspring meiotic recombination and life history, both of which have the potential to accelerate adaptive evolution. Taken together, these results demonstrate the benefits of integrating life history theory in eco-immunological research. They show that life history responses are an integral component of host defence against parasites, and that *Drosophila-Metarhizium* is a promising model system for ecological immunology.

Chapter 1 General Introduction

1.1 Ecological Immunology

The study of host defence against parasites and pathogens has traditionally been carried out by immunologists. However, a rapidly growing field has emerged over the past two decades that provides a complimentary approach to classic immunology by incorporating concepts and methodologies from evolutionary ecology. Coined 'ecological immunology', this new field attempts to understand the ecological and evolutionary drivers that maintain the pervasive variation in immune defence observed in both the laboratory and the field (Sheldon & Verhulst 1996).

The growth and development of ecological immunology has been reviewed extensively (Rolff & Siva-Jothy 2003; Schmid-Hempel 2003; Schulenburg et al. 2009; Martin et al. 2011). One of the most important advances brought on by an eco-immunological approach is the realization that host immunity is constrained by trade-offs with other fitness components. Given limited resources, an individual that invests heavily in immunity has to make sacrifices elsewhere e.g. a reduction in growth or reproduction (Stearns 1992). Activating the immune system in response to parasites incurs additional costs of deployment through increased resource allocation and autoimmunity (Moret 2000; Sadd & Siva-Jothy 2006). Over evolutionary time, such trade-offs can result in negative genetic covariance between immunity and other fitness traits (Schmid-Hempel 2003; McKean et al. 2008). Thus, paradoxically, selection might favour imperfect defence against parasites because it optimally balances the costs and benefits of immunity.

1.2 Current challenges

Despite the tremendous advances in our understanding of how animals resist the spread of infectious agents and why variation in host immunity persist, ecological immunologists still face a number of conceptual and methodological challenges.

1.2.1 Life history strategies and fitness measures

Although life history theory lies at the heart of an eco-immunological approach to the study of host immune defence, the integration of life history response and

immune defence remains poorly understood (Schulenburg et al. 2009). Firstly, while mounting immune response can result in trade-offs with life history traits (cost of immune deployment), life history changes as a result of parasite exposure can also represent host defence strategy in their own rights (Forbes 1993; Minchella 1985; Agnew et al. 2000; Hurd 2001). However, few eco-immunology studies have addressed this possibility or its implications for other components of host immune defence.

Secondly, while the need to measure fitness empirically has been recognised as a key component in the design of eco-immunological studies (Graham et al. 2010), it is less clear how it should be achieved. Competitive ability and survival have been measured to demonstrate the fitness costs of immunity (Kraaijeveld & Godfray 1997; Moret 2000; Armitage et al. 2003; Schmid-Hempel 2003), though relatively few studies have measured reproductive fitness. When reproductive fitness have been measured directly, often lifetime reproductive success is reported (e.g. McKean et al. 2008). However, the appropriate fitness measure depends on the demographic context: early-age reproduction matters the most in expanding populations, while late-age reproduction is more important in declining populations (Charlesworth 1994; Brommer 2000). While useful for providing inferences in stable population, LRS is insensitive to the timing of reproduction. In contrast, the intrinsic rate of increase captures reproductive timing, a key life history trait, could provide novel insights in studies of immunity. A final complication is that fitness measures within a single generation may not be indicative of inclusive fitness across multiple generations, which could challenge the established interpretation of previous findings (Priest et al. 2008a; Priest et al. 2008b).

1.2.2 Environmental dependence of immunity

It has become increasingly apparent that the functioning of immune defence is highly dependent on the environmental context (reviewed in Lazzaro and Little 2009). Biotic and abiotic factors such as temperature (Thomas & Blanford 2003), diet (Siva-Jothy & Thompson 2002; Lee et al. 2008), crowding (Barnes & Siva-Jothy 2000; Wilson et al. 2002), and mating (Lawniczak et al. 2007; Morrow & Innocenti 2012) all impact on immune functions. Although frequently excluded from controlled experiments as troublesome sources of variation or 'noise', these environmental heterogeneities may play an important role in maintaining genetic

polymorphisms in immune functions (Lazzaro & Little 2009). Hosts could actively exploit such environmental dependence to augment their immune defence e.g. through behavioural thermoregulation (Thomas & Blanford 2003). Potential hosts may also use environmental cues that reliably predict infection risk to mount pre-emptive immune defence e.g. immune anticipation of mating (Siva-Jothy 2009). Similarly, maternal parasite exposure history could predict the parasites that her offspring are likely to encounter and selection may favour transgenerational immune priming (e.g. Little et al. 2003; Yannick Moret 2006; Ben M Sadd and Schmid-Hempel 2007). However, we do not know if parental effects of parasite exposure also influence offspring traits such as life history and meiotic recombination, which could have significant ecological and evolutionary implications (Kirkpatrick & Lande 1989; Agrawal et al. 2005).

1.2.3 Methodological critiques

There have been growing recognitions of the limitations inherent in many of the common methods and approaches employed in eco-immunological studies. Firstly, immune function measurements should be interpreted with caution (Adamo 2004; Viney et al. 2005). Measures of individual components of immunity, such as PO activity or haemocyte counts, are unlikely to be accurate indicators of overall 'immuno-competence' because different arms of the immune system may trade off with one another (Cotter et al. 2003), and because higher measurements do not necessarily indicate greater pathogen resistance; in fact, the reverse may often be true (Auld et al. 2012). More recently, advances in molecular techniques have enabled the routine measurements of gene expressions for hundreds of putative immune genes in model species such as *Drosophila melanogaster* (De Gregorio et al. 2001; Roxström-Lindquist et al. 2004). However, for the majority of such immune-related genes their functional significance is unknown; indeed, higher 'potential' immune response as measured by gene expressions in *Drosophila* has been shown to translate into lower 'realized' immune response as measured by survival post-bacterial challenge (Fedorka et al. 2007).

A second major limitation is that many eco-immunological studies in insects have used techniques, such as piercing with a needle, that physically disrupt the integument to introduce general immune elicitors (e.g. lipopolysaccharide), non-pathogenic bacteria (e.g. *Escherichia coli*) or foreign objects (e.g. nylon rods)

directly into the body (e.g. Siva-Jothy, Tsubaki, and Hooper 1998; McKean and Nunney 2001; Fedorka et al. 2007). While useful as a means to obtain standardized measures of immune response, such assays are particularly physically damaging for small insects, which can mask biologically relevant but more subtle effects on host immunity (Wigby et al. 2008). Furthermore, by completely bypassing barrier defence and other defence mechanisms such as behaviour, this can lead to an overestimation of the importance of humoral and cell-mediated immune defence (Schmid-Hempel & Ebert 2003; also see **Figure 1.1**). Lastly, given the recent evidence for strong specificity in invertebrate immune responses (Little et al. 2005; Schmid-Hempel 2005; Schulenburg et al. 2007), it is unclear the extent to which experiments using generalised immune elicitors and non-pathogenic bacteria can inform us about the interactions between the host and its natural pathogens.

1.3 *Metarhizium* and *Drosophila* as a model system

A promising laboratory model for ecological immunology of insects is the fruit fly *Drosophila melanogaster* and the entomopathogenic fungus *Metarhizium robertsii*. *D. melanogaster* has been a key model species for the study of innate immune system (Lemaitre & Hoffmann 2007). A wealth of molecular and genetic tools are available in *Drosophila* that enable detailed dissections of immune mechanisms (e.g. a genome-wide RNAi library, see Dietzl et al. 2007). With short generation time and high fecundity, the fruit fly is also amenable to large-scale demographic and life history studies, which are needed to address questions of adaptive significance (Prasad & Joshi 2003). By contrast, entomopathogenic fungi have received less attention from eco-immunologists. Here, I provide a brief overview of their ecology, pathogenesis mechanisms and host defences against them.

1.3.1 Distribution and ecology of entomopathogenic fungi

Encompassing more than 700 species, entomopathogenic fungi are a major factor in the regulation of natural insect populations (Roberts & St Leger 2004). *Metarhizium spp.*(Ascomycota, Hypocreales) are primarily clonally-reproducing fungi with a global distribution in natural and cultivated soils, which have been developed as a model for insect-fungal interaction with extensive applications in biocontrol (St Leger 2007). Many strains within the genus such as *M. robertsii* (previously

classified *M. anisopliae* strain ARSEF 2575; Bischoff et al 2009), are generalist pathogens with a broad host spectrum. There are also host-specific specialist strains, which are thought to have derived repeatedly from generalist strains through the loss of virulence-genes (Wang et al. 2009). While entomopathogenic fungi were first identified by their insecticidal properties, recent advances in fungal ecology suggest that they also play additional roles in natural populations (Vega et al. 2009). For example, *Metarhizium* has been shown to be rhizosphere competent and capable of persisting in the environment for extended amounts of time (Hu & Leger 2002). Recent evidence suggests that the symbiotic relationship between *Metarhizium* and plants are important for soil-nitrogen cycling (Behie et al. 2012).

1.3.2 Pathogenesis mechanisms

The success of entomopathogenic fungi can be partially attributed to their unique mode of infection. Unlike bacterial or viral infections, successful establishment of fungal infection do not require ingestion or external injuries of the cuticle. Instead, all entomopathogenic fungi produce infective spores (conidia), which germinate and directly penetrate the cuticle.

The infection process is best understood in *Metarhizium* (reviewed in Clarkson and Charnley 1996). Facilitated by a specialised adhesion molecule MAD1 (Wang & St Leger 2007), conidia first attach to the hydrophobic surface of the insect epicuticle. Germination then occurs under high humidity forming the infection structure (appressorium), which along with penetrating hyphae, produce and secrete a host of cuticle-degrading enzymes including the potent serine endoprotease PR1 (Goettel & Leger 1989). After breaching the cuticle, *Metarhizium* proliferate in the haemolymph first as single-celled wall-less protoplasts, and later as multi-celled walled blastospores and hyphal bodies. During this yeast-like stage, fungal cells evade detection by host haemocytes through concealing antigenic structures within a protective collagenous coat (Wang & St Leger 2006). Moreover, *Metarhizium* actively produce a battery of toxins including Destruxin A, which actively suppresses the induction of antimicrobial peptides (AMPs), a key host humoral defence mechanism (Pal et al. 2007), and may also inhibit behavioural defence mechanisms including fevering (Hunt & Charnley 2011). After host death, the fungus grows saprophytically culminating in extensive growth of hyphae on the cadaver and the production of vast numbers of conidia.

1.3.3 Antifungal immunity

To mitigate the detrimental effects of fungal infection, insects have evolved a range of defence mechanisms. Traditionally, immunologists have naturally focussed on the role of the innate immune system; so called because insects lack the antibody-based acquired immune system that characterise vertebrates (Gillespie et al. 2000; Levitin & Whiteway 2008). Prior to the activation of humoral and cellular immune responses, fungal infections in *Drosophila* are detected via two complimentary pathways: recognition of fungal cell wall components (β-1,3-glucans) by the pattern recognition receptor Gram-negative binding proteins 3 (GNBP-3) and activation of the protease Persephone (psh) by fungal virulence factors (Gottar et al. 2006). Both pathways lead to the activation of the Toll pathway, which result in the rapid synthesis of antifungal AMPs including *Drosomycin* and *Metchnikowin* by the fat body (Lemaitre & Hoffmann 2007; Levitin & Whiteway 2008). In general, Toll pathway mutants show increased susceptibility to both natural and artificial fungal infections (Lemaitre & Hoffmann 2007).

The humoral responses are complimented by haemocyte-based cellular immune responses. Some 95% of *Drosophila* haemocytes are plasmatocytes that function in phagocytosis, while the remaining 5% are crystal cells, which are involved in the activation of prophenoloxidase (proPO) cascade leading to the production of quinones and melanin (Grell et al. 1980). However, recent studies suggest that insect cellular immune responses may be largely ineffective against entomopathogenic fungi. Haemocytes are poorly attracted to fungal cells in the haemolymph and even those fungal cells that are phagocytised continue to grow within host cells (Gillespie et al. 2000; Wang & St Leger 2006). Similarly, activation of proPO cascade does not appear to be critical for survival post-fungal infections in *Drosophila* (Leclerc et al. 2006; Tang et al. 2006).

1.4 Thesis overview

How do animals defend themselves against parasites? What ecological and environmental factors have shaped their defence mechanisms? Do infections have consequences for hosts that extend beyond a single generation? What are the likely evolutionary consequences of such transgenerational effects? Using *Metarhizium* and *Drosophila* as a model, I have attempted to tackle these questions during my PhD by

combining life history, eco-immunological, population and molecular genetics approaches. A common theme uniting the chapters is the plasticity of life history, which has implications for how organisms maintain and deploy the immune system, and how we measure the fitness consequences of immunity.

Following the defence component model proposed by Schmid-Hempel and Ebert (2003; **Figure 1.1**), I first examine the pre-infection stage often ignored in eco-immunological studies. Successful infection by parasites requires first encountering susceptible hosts who could protect themselves by avoiding coming into contact with parasites. Alternatively, if encounters are unavoidable but predictable such as acquiring sexually transmitted infections during mating, they could anticipate the immune challenge and pre-emptively activate immune defences. In **Chapter 2**, I examine this second possibility and provide empirical support for the hypothesis of immune anticipation of mating using an RNA interference genetic system in *Drosophila* and experimental sexual transmission of *Metarhizium*. I also establish the functional significance of immune and stress response gene expression and identify life history costs of immune gene expression, a central prediction in ecological immunology.

Once host avoidance has been overcome and the initial infection has been established, the host can employ immunological, behavioural and life history responses to minimise the fitness costs of infection. In **Chapter 3**, I show that host responses are integrated to act synergistically: parasite-induced thermoregulatory behaviour, instead of simply extending host survival, also facilitate an adaptive switch in the host life history strategy. While the trade-offs between immune function and fitness components are sometimes seen as essentially static within individuals (e.g. negative genetic covariance or the 'evolutionary cost' of immunity; Schmid-Hempel 2003), a more accurate view might be that host life history has evolved to be highly flexible and capable of rapid adjustments to suit the prevailing ecological context.

If a host survives an infection or is able to achieve some reproductive success before dying, then opportunities exist for the parental generation to pass information regarding the parasite environment to their progeny. Moreover, individuals are likely to differ in their ability to transmit these transgenerational effects based on their fitness or genetic quality. In **Chapter 4**, using classic fruit fly population genetics in a fully factorial experimental design across three generations, I demonstrate that

fungal infections can induce fitness-associated maternal effects, which alter both the life history and meiotic recombination rates in the offspring. These results are significant because population and quantitative genetics models predict that such transgenerational effects have the potential to accelerate the rate of adaptive evolution in parasite-rich environments (Kirkpatrick & Lande 1989; Agrawal et al. 2005).

In **Chapter 5**, I draw together common themes from Chapter 2-4 and briefly discuss their implications for ecological immunology. I also highlight promising directions for future research.

Finally, I have also worked on another interesting evolutionary problem during my PhD: the controversial concept of evolvability and the empirical methods with which it could be tested. This work has resulted in a published manuscript (Zhong & Priest 2010), which is attached in **Appendix I**.

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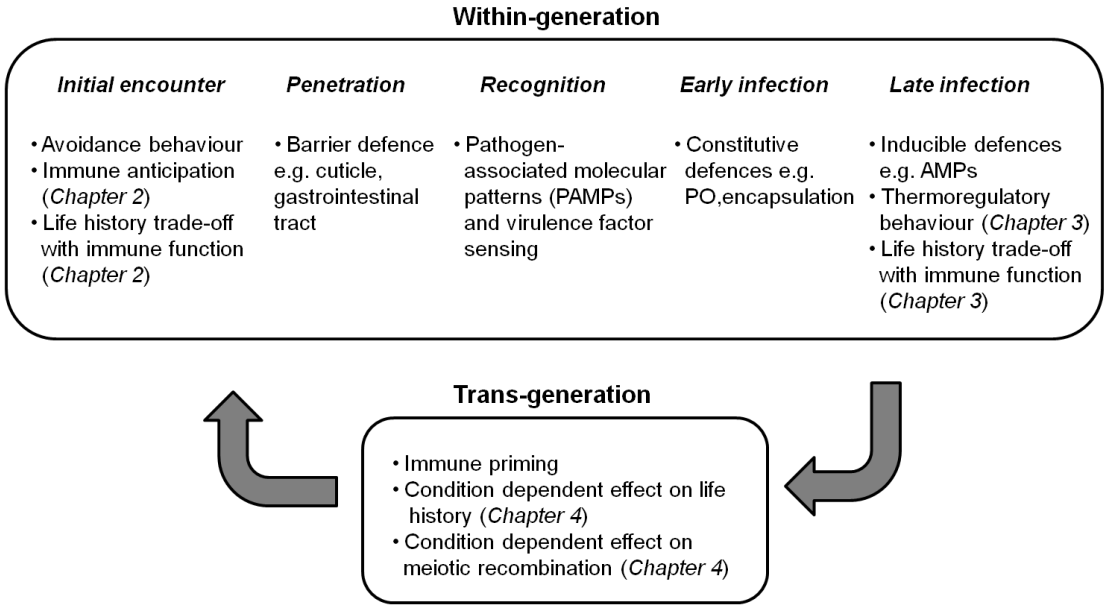

Figure 1.1 An 'extended' defence component model illustrating potential *Drosophila-Metarhizium* interactions (modified from Schmid-Hempel & Ebert 2003). Successful infection depends on sequentially overcoming host defence at each infection stage (also see 'steps of infection model', Duneau et al. 2011). I have focussed on 'non-immunological' defence mechanisms including life history and behavioural responses to infections, which have recently attracted extensive interests in the literature (Thomas & Blanford 2003; Parker et al. 2011; Baucom & de Roode 2011; de Roode & Lefèvre 2012). I extend the model to incorporate potential transgenerational effects of pathogen exposure such as TGIP (e.g. Little et al. 2003; Moret 2006; Sadd & Schmid-Hempel 2007).

Chapter 2 Immune anticipation of mating in *Drosophila*: *Turandot M* promotes immunity against sexually transmitted fungal infections

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Abstract

Although it is well known that mating increases the risk of infection, we do not know how females mitigate the fitness costs of sexually transmitted infections (STIs). It has recently been shown that female fruit flies, *Drosophila melanogaster*, specifically upregulate two members of the *Turandot* family of immune and stress response genes, *TotM* and *TotC*, when they hear male courtship song. Here we use the *Gal4/UAS* RNAi gene knockdown system to test whether the expression of these genes provides fitness benefits for females infected with the entomopathogenic fungus, *Metarhizium robertsii* under sexual transmission. As a control, we also examined the immunity conferred by *Dif*, a central component of the *Toll* signalling pathway thought to provide immunity against fungal infections. We show that *TotM*, but not *TotC* or *Dif*, provides survival benefits to females following STIs, but not after direct topical infections (DTIs). We also show that though the expression of *TotM* provides fecundity benefits for healthy females, it comes at a cost to their survival, which helps explain why *TotM* is not constitutively expressed. Together, these results show that the anticipatory expression of *TotM* promotes specific immunity against fungal STIs, and suggest that immune anticipation is more common than currently appreciated.

Author contribution:
WZ and NKP designed the experiments and prepared the manuscript; WZ, CDM, CRE and DTM conducted the experiments; WZ performed the analysis; EI and MGR provided expression data and improved the manuscript

2.1 Introduction

Mating is fraught with danger. In addition to the fitness costs associated with finding sexual partners, copulation and offspring production, mating increases the risk of acquiring sexually transmitted infections (Sheldon 1993; Lockhart et al. 1996; Knell & Webberley 2004). In insects, STIs are often both highly prevalent and pathogenic (Knell & Webberley 2004; Webberley et al. 2006). It is generally thought that they exert a selective pressure strong enough to influence the evolution of mating systems, life histories, sexual conflict and sexual behaviour (Kokko et al. 2002; Knell & Webberley 2004). Yet, we have a poor understanding of how they have shaped the immune system (Wlasiuk & Nachman 2010).

Females might mitigate the risks of acquiring STIs through immune anticipation of mating, the activation of immune responses before sexual congress and potential exposure to pathogens (Siva-Jothy 2009). Pre-emptive immune activation is predicted to be more advantageous than a purely reactive response because it shortens the time delay of the immune response, and thereby maximises its efficiency (Siva-Jothy 2009). We know that females upregulate a number of immunity-related genes in response to mating (Lawniczak & Begun 2004; McGraw et al. 2004; Mack et al. 2006; McGraw et al. 2008; Domanitskaya et al. 2007). However, even the act of courtship might stimulate immune activation. If immune genes expressed during courtship represent immune anticipation of mating, then we would expect such responses to enhance immunity against STIs and to exhibit costs in some aspects of life history, because otherwise they would be expressed constitutively (Sheldon & Verhulst 1996; Schmid-Hempel 2003).

One way to address this possibility is to identify candidate immune genes associated with courtship, and perform infection and fitness assays in which the expression levels of the genes are manipulated. Recently, *Turandot C* and *Turandot M* (*TotC* & *TotM*), members of the *Turandot* family of immune and stress response genes, were shown to be upregulated in the heads of female *D. melanogaster* stimulated by male courtship songs, independent of any physical contact with males (Immonen & Ritchie 2012). Of the two, *TotM* is likely to be the better candidate for anticipatory immunity against STIs, as it is poorly induced by non-immune related stress (Ekengren & Hultmark 2001), but strongly induced by both fungal infections (Ekengren & Hultmark 2001; De Gregorio et al. 2001; Roxström-Lindquist et al.

2004) and mating (McGraw et al. 2004; Innocenti & Morrow 2009; Gioti et al. 2012). In addition, induction of *TotM* by natural fungal infection exhibits similar fold-change in expression to well-known antifungal antimicrobial peptides (AMPs) including *Drosomycin* and *Metchnikowin* (Roxström-Lindquist et al. 2004). Surprisingly, there is little evidence that courtship stimulates the upregulation of the canonical *Toll* and *Imd* pathway immune genes, such as *Dorsal-related immunity factor* (*Dif*), a NF-κB- like factor that regulates *Toll*-dependent immune responses thought to provide immunity specifically against gram-positive bacteria and fungi (Lemaitre et al. 1996; Rutschmann et al. 2000).

Previous efforts in establishing *Drosophila melanogaster* as a model laboratory system for studying insect STIs have focused on bacterial pathogens (Miest & Bloch-Qazi 2008; Gendrin et al. 2009; Radhakrishnan & Fedorka 2012). However, entomopathogenic fungi might be more appropriate as a model for sexually transmitted parasites. First, entomopathogenic fungi are widespread across diverse environments causing a large proportion of all known insect STIs and indeed, the majority of all insect diseases (Knell & Webberley 2004; Roberts & St Leger 2004). Second, because fungal spores cause infection through direct contact with the cuticle (Clarkson & Charnley 1996; Roy et al. 2006), they are amenable for comparisons between sexual and non-sexual horizontal transmission. Finally, studying the sexual transmission potential of entomopathogenic fungi in the laboratory have important implications for their application in the field as agents of biocontrol (Meadow et al. 2000; Toledo et al. 2007; Dimbi et al. 2013).

Here, we examine the hypothesis that *TotM* provides protection against sexually transmitted *Metarhizium robertsii*, a generalist entomopathogenic fungus, which exhibits both sexual and non-sexual transmission in dipterans and has been used extensively in biocontrol (Roberts & St Leger 2004; Dimbi et al. 2009; Gao et al. 2011). Here, we test the predictions that 1) *Metarhizium* can be sexually transmitted in *D. melanogaster*, that 2) expression of *TotM* helps to mitigate the cost of infections under sexual transmission, but not direct modes of transmission, and that 3) the expression of *TotM* has fitness costs in the absence of *Metarhizium* STIs. To address these questions, we use the Gal4/UAS RNAi targeted gene knockdown approach (Duffy 2002), in conjunction with large-scale demographic analysis, to

estimate the immunity and fitness conferred by *TotM*, *TotC* and *Dif* under both STIs and high-dose direct topical infections (DTIs) of *M. robertsii*.

2.2 Materials and Methods

2.2.1 Fly strains and fungal culture maintenance

A wild-type Dahomey strain of *D. melanogaster* (provided by Dr Stuart Wigby, University of Oxford) was kept in large population cages ($1m^3$) with overlapping generations for two years prior to the start of the experiments. RNAi strains were obtained from Vienna Drosophila RNAi Center (UAS-*TotM*, transformant ID 106727; UAS-*TotC*, transformant ID 106379; UAS-*Dif*, transformant ID 30579, see Dietzl et al. 2007). We used the non-tissue specific Act5C promoter to drive ubiquitous expression of Gal4 and UAS constructs (Act5C-Gal4/CyO, Bloomington Stock Centre stock number 4414). We crossed Act5C-Gal4/CyO females with males carrying one of the UAS constructs to generate the active knockdown genotypes (Act5C-Gal4/UAS-*TotM*-IR; Act5C-Gal4/UAS-*TotC*-IR; Act5C-Gal4/UAS-*Dif*-IR). As a control for the presence of the UAS transgene, we crossed w1118 wild-type females (the genetic background for all RNAi lines, obtained from Bloomington Stock Centre) with males carrying one of the UAS constructs (UAS-*TotM*-IR/+; UAS-*TotC*-IR/+; UAS-*Dif*-IR/+). As a control for the presence of the Gal4 driver, we crossed Act5C-Gal4/CyO females with w1118 males (Act5C-Gal4/+). The effectiveness of RNAi knockdowns of *TotM* and *TotC* was confirmed by semi-quantitative PCR (Immonen 2012). All experimental animals were maintained at 25°C with 12:12 light-dark cycle in standard *Drosophila* vials at low densities (~50 flies/vial) for at least two generations prior to the start of experiments. We used an oatmeal-molasses-agar media with added live baker's yeast and an antifungal agent (Nipagin), which inhibited the growth of naturally-occurring saprophytic fungi. All experimental flies used were collected as virgins over a period of 24 hours.

Metarhizium robertsii (isolate 2575, previously known as *M. anisopliae* Strain ME1) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF, United States Department of Agriculture). We inoculated quarter-strength Sabouraud Dextrose Agar with additional yeast extract (SDAy) with *M. robertsii* conidia (asexual fungal spores) and incubated the plates at 28°C for four weeks before storing at 4°C for up to three

months. Conidia were collected by scraping the surface of the sporulating culture with an inoculating loop.

2.2.2 Sexual transmission of fungal pathogen

We assessed the transmission potential of *M. robertsii* by exposing healthy Dahomey females to males that had been topically inoculated with the fungus. At adult age day 4, groups of ten virgin males were topically inoculated with 6 mg of conidia without CO_2 anaesthesia by shaking in a 250ml conical flask for 20 seconds. Inoculated flies were held in temporary holding vials for 24 hours, ensuring that they had opportunities to groom themselves, which has previously been shown to be effective at removing fine dust particles (Phillis et al. 1993). At adult age day 5, each infected male fly was introduced into a new vial containing 10 uninfected virgin females of the same age and removed after 24 hours. The logic of giving males time to groom and subsequently using a fresh vial is to allow male to adopt a more natural behaviour (Dimbi et al. 2009) and to minimise the chance of females contracting infection from conidia that had been dislodged during grooming. We then transferred and held treated females in individual vials for a further 24 hours to allow egg-laying. The presence of larvae four days after oviposition indicated that the female had mated with infected male. We assessed the infection status of females by the presence of *Metarhizium*-like fungal growths on cadavers. After brief immersion in 70% ethanol, flies were gently crushed and placed in Petri dishes on moistened filter paper at the end of the egg-laying period. After an incubation period of 5 days at 28ºC, we examined all cadavers for signs of *Metarhizium*-like fungal growth (either hyphae or conidia) with a low-power dissection microscope. Because high levels of horizontal transmission of conidia between infected and naïve files due to non-sexual contact could confound our interpretation, we also assessed the non-sexual horizontal transmission potential of *M. robertsii* using the same procedures described above by exposing naïve males and females to infected flies of the same sex.

2.2.3 Survival assays under DTI and STI

We assessed the effects of gene knockdowns on survival under high-dose direct topical infections and sexual transmission using adult flies for all genotypes. For DTI, at adult age day 7, we infected groups of approximately 300 mixed sex flies of each genotype with 20mg of conidia, or kept as uninfected control, following the protocol

described previously. Inoculated flies were held in temporary holding vials for 30 minutes before being transferred to demography cages (10x15cm). For STI, we first inoculated 6-day old w1118 males in groups of 20 with 12mg of conidia, and then transferred 20 infected or control males with 20 uninfected females to demography cages at adult age day 7. As infected males in STI treatment suffered much greater mortalities than control males, we restored the original complement of 20 infected males by adding freshly infected w1118 males at day 12 and 24 post-inoculation. For both DTI and STI, we removed and recorded dead flies daily until day 9 post-inoculation and every two days thereafter. We also tracked the changes in pathogen loads in the first 24 hours following DTI by sampling inoculated Dahomey wild-type flies at three time points post-inoculation (0 hour, 2.5 hours and 24 hours; n=9). Sampled flies were individually homogenised in 200µl of 0.04% Tween80®, diluted by a factor of 10^3 and spread onto standard SDAy plates. Pathogen loads were assessed by counting the numbers of colony forming units (CFUs) following incubation at 28°C for 24 hours.

2.2.4 Fecundity assay under STI

We assessed the effects of gene knockdowns on survival and fecundity of females exposed to fungus-infected males using flies from the same cohort collected for survival assays. In the fecundity assay, we first infected two-day old w1118 wild-type adult males (the genetic background of RNAi strains). At 24 hours-post inoculation, infected or uninfected control males were transferred to individual vials containing a single uninfected virgin female for each genotype. The mating pairs were assigned positions in randomised blocks and transferred to new vials after 24 hours, and thereafter every two days until day 9 (n=55/treatment/genotype). Used food vials were frozen 18 days after collection and the numbers of hatched pupae were counted giving a combined measure of fecundity and larval viability. We assessed the proportion of females that became infected through mating with infected males by sampling all surviving females at the end of day 9 post inoculation (96.8%, 701/724) and checking for signs of *Metarhizium*-like fungal growth after incubation at 28°C for up to two months, following the protocol described previously.

2.2.5 Statistical analysis

All statistical analyses were performed with R version 2.15 (R Core Team 2012). We assessed the contribution of mating to the transmission of STIs by comparing the proportions of flies that displayed *Metarhizium*-like fungal growth for mated females, and those that were kept with infected males but remained virgin using chi-square tests with continuity correction. We used student's t test on CFUs to directly compare pathogen loads immediately after inoculation and after 24 hours.

Cox proportional hazard regressions were used to analyse all survival data. The full model (including all genotypes) contained age at death and censoring information as the response variables, and genotype, infection treatment and their interaction as predictor variables. A separate Cox regression was performed for each gene of interest that only included the relevant knockdown and control genotypes (e.g. *TotM*: Act5C-Gal4/UAS-*TotM*, Act5C-Gal4/+ and +/UAS-*TotM*). For each gene of interest, we first extracted the hazard ratios (the fold-increase in risk of death in infected animals relative to uninfected controls) for the knockdown genotype and its combined control genotype (by pooling raw survival data of the relevant control genotypes) from the Cox models. Because the mortality rate in the DTI treatment is substantially higher than in the STI treatment, it is difficult to directly compare the effect of immune gene knockdowns in the two treatments. To overcome this problem, we calculated normalized hazard ratios by dividing the hazard ratios of each knockdown by its associated combined control genotype. Unlike simple metrics of lifespan, this measure describes the effect of each gene knockdown on immunity normalized for its genetic background, which allows us to directly compare the immune properties conferred by genes under STIs and DTIs, despite great differences in effect size. We assessed the survival cost of gene expression in the absence of infections by comparing the hazard ratios of each gene knockdown relative to its combined control genotype under uninfected control conditions.

We used mixed effects models to assess the effects of genotype and infection on fecundity across time. The full model included the number of hatched pupae produced at each time point as the response variable; genotype, treatment, time and all associated two-way interactions as fixed effects (three-way interaction was non-significant when fitted and thus dropped from the full model), and individual females as random effect (intercepts). We also included the age at death of male partners as a covariate in the full model to account for the possibility that females might have lower fecundity under STI simply due to a lack of remating

opportunities as infected males die at earlier ages than uninfected controls. Female fecundity in the first 24 hours was excluded from the model as the fecundity was much lower than at other time points and further experiments suggested minimal *in vivo* fungal growth in this period (Chapter 3). We assessed the fecundity cost of gene expression in the absence of infections by comparing the mean total pupae productions of the gene knockdown (day 0-9 post treatment) and the combined control genotype using one-way analysis of variance.

2.3. Results

2.3.1 Sexual transmission of fungal pathogen

We found that *Metarhizium robertsii* can be sexually transmitted in the fruit fly, with approximately one in five (55/263) naïve females displaying *Metarhizium*-like fungal growth on their cadavers after being placed with a topically infected male for 24 hours (**Figure 2.1a** and **b**). Further analysis showed that fungal transmission was driven primarily by mating, as the proportion of cadavers with fungal growth was higher in gravid females than infertile females (χ^2_1=8.96, p=0.0028; **Figure 2.1c**). The dose received by females was likely to be low as the pathogen load of the topically infected males was only ~5,000 CFU, which had declined by grooming from the initial load of ~20,000 CFUs (t=7.69, p=0.006; **Figure 2.2**). Finally, we also found that *Metarhizium* could be transmitted among same sex flies (7/277 for male to male and 7/266 for female to female; **Figure 2.3**). Nevertheless, naïve flies were much more likely to be infected through sexual transmission than non-sexual transmission, 20.9% vs. 2.6%, respectively.

2.3.2 Effects of STI and DTI on survival across RNAi strains

We found that *TotM* promotes immunity against *Metarhizium* when it is sexually transmitted (STI), but not when it is applied as a direct topical infection (DTI). The effect of STIs on the hazard ratio, which estimates the risk of death in infected treatments relative to control treatments, was highly dependent on the host genotype (Overall: Genotype × Treatment, χ^2_6=26.4, p=0.0002; **Figure 2.4a**). Specifically, *TotM* knockdown flies (Act5c-Gal4/UAS-*TotM*-IR) were susceptible to STIs but there was no evidence of susceptibility in either of +/Act5c-Gal4 or +/UAS-*TotM*-IR control genotypes (Genotype × Treatment; χ^2_2=15.8, p=0.00037; **Figure 2.4a**). In

contrast, there was no variation in susceptibility with respect to Dif knockdown flies (Act5c-Gal4/UAS-*Dif*-IR) or either of its +/Act5c-Gal4 and +/UAS-*Dif*-IR control genotypes (Genotype × Treatment, χ^2_2=0.05, p=0.98). Surprisingly, *TotC* knockdown flies (Act5c-Gal4/UAS-*TotC*-IR) had higher survival post-infection than both of its control +/Act5c-Gal4 and +/UAS-*TotC*-IR genotypes (Genotype × Treatment, χ^2_2=9.1, p=0.011; **Figure 2.4a**).

We found different patterns under direct topical infection. While DTIs generally caused very rapid mortalities such that 95% of flies died within 9 days, some genotypes were much more susceptible (Overall: Genotype × Treatment, χ^2_6=751, p<0.0001; **Figure 2.4b**). As expected (Rutschmann et al. 2000), *Dif* knockdown (Act5c-Gal4/UAS-*Dif*-IR) females were significantly more susceptible to DTIs than either of its control genotypes (Genotype × Treatment, χ^2_2=545, p<0.0001; **Figure 2.4b**). However, neither *TotM* nor *TotC* knockdowns were more susceptible to DTIs than their respective control genotypes (**Figure 2.4b**). Interestingly, although the hazard ratio of the *Dif* knockdown line under DTI was more than 16 times higher than that of *TotM* knockdown under STIs (46.2 ± 6.2 vs 2.8 ± 0.6), their hazard ratios were comparable after they were normalized to account for the susceptibility of their control genotypes (2.7 ± 0.7 vs 2.4 ± 0.4; **Figure 2.4c**).

2.3.3 Effect of STI on fecundity across RNAi strains

Sexually transmitted *Metarhizium* infections resulted in reproductive costs for female flies. Exposure to topically infected male partners initially had little impact on female reproduction, but over time, female fecundity in the infected treatment declined relative to uninfected controls (Treatment × Time, $F_{1,2030}$=30.3, p<0.0001; **Figure 2.5**). The reduction in female fecundity under STIs could not be explained by a lack of remating opportunities due to mortalities of infected male partners, because male longevity did not significantly contribute to female fecundity over the course of the experiment ($F_{1,705}$=3.5, p=0.062). This pattern was apparent in all lines as there was no evidence that *TotM* or indeed any gene knockdown strain suffered greater fecundity reduction than their control genotypes (Treatment ×Genotype, $F_{6,705}$=1.45, p=0.19). In addition, while the cadavers of females that have been exposed to infected males were more likely to exhibit *Metarhizium-like* fungal growth (χ^2_1=5.69, p=0.017) than cadavers of females exposed to control males, there was no evidence

that the RNAi knockdown genotypes influenced the probability of fungal growth (χ^2_1=0.001, p=0.97; **Figure 2.6**).

2.3.4 Effect of gene expression on survival and fecundity in uninfected flies

We found that the expression of *TotM* and *Dif*, but not *TotC*, results in survival costs for uninfected females. Both *TotM* and *Dif* knockdown flies (Act5c-Gal4/UAS-*TotM*-IR and Act5c-Gal4/UAS-*Dif*-IR), but not *TotC* knockdown flies (Act5c-Gal4/UAS-*TotC*-IR), showed enhanced survival relative to their control genotypes (*TotM*: χ^2_1=8.58, p=0.0034; *Dif*: χ^2_1=26.6, p<0.0001; *TotC*: χ^2_1=0.88, p=0.35; **Figure 2.7a**). In contrast, we found evidence for reproductive benefits of *TotM* and *TotC* expression, but reproductive costs of *Dif* expression. Both *TotM* and *TotC* knockdown females had lower total reproduction than their respective controls, while that for *Dif* knockdown females was higher than its control genotypes (*TotM*: $F_{1,135}$=44.8, p<0.0001; *TotC*: $F_{1,127}$=7.6, p=0.0068; *Dif*: $F_{1,129}$=6.3, p=0.014; **Figure 2.7b**).

2.4 Discussion

Mechanisms of insect immunity are known to be pathogen-specific (Lemaitre et al. 1997; Lemaitre & Hoffmann 2007); but, the extent to which insects use ecological cues to inform which responses to mount is not known. Our study shows that a gene that is upregulated in anticipation of mating provides protection against the entomopathogenic fungus *Metarhizium* when it is sexually transmitted. This finding is important because it illuminates the molecular mechanisms and life history costs and benefits which underlie immunity against STIs. In combination with previous results (Immonen & Ritchie 2012), our results imply that fruit flies demonstrate immune anticipation of mating and that immune anticipation could be a general mechanism for achieving immune specificity.

2.4.1 A Turandot gene that enhances immunity against STIs

Hundreds of *Drosophila* genes, including *TotM*, have been indentified on the basis of expression induction following immune challenges, but the functional consequences of these genes are rarely established (Ekengren & Hultmark 2001; De Gregorio et al. 2001; Roxström-Lindquist et al. 2004). This is a problem because

gene expression does not necessarily translate to immunity against live pathogens (Adamo 2004; Fedorka et al. 2007; Lawniczak et al. 2007). We show that *TotM* confers protection to fungal STIs *in vivo*, and its effects are similar in magnitude to that conferred by *Dif* to fungal DTIs.

The mechanisms through which *TotM* enhances immunity are currently unknown. All protein products encoded by the *Turandot* gene family are thought to be actively produced in the *Drosophila* fat bodies and secreted into the haemolymph, where they are hypothesized to act as protein chaperones or as signalling molecules (Ekengren et al. 2001; Ekengren & Hultmark 2001). Though direct tests are needed, it seems unlikely that *TotM* possesses direct antimicrobial activities similar to known antifungal AMPs such as *Drosomycin* and *Metchnikowin*, since over-expression of another *Turandot* gene, *TotA* does not provide increased protection against gram-negative bacterial infections (Ekengren et al. 2001; Agaisse et al. 2003). Instead, *TotM* might help the fly to tolerate persistent fungal infections by mitigating the negative effects of the infection without actively suppressing pathogen growth (Baucom & de Roode 2011; Medzhitov et al. 2012; Ayres & Schneider 2012). Consistent with a role in enhancing tolerance, not resistance, we found that fungi were as likely to emerge from the control genotype flies as they were from *TotM* knockdown flies.

2.4.2 Mode of transmission and immunity

Fruit flies have a remarkable ability to mount immune responses which are specific to the pathogen (Lemaitre et al. 1997; Lemaitre & Hoffmann 2007). Our work shows that the efficacies of their immune responses are also specific to the mode of infection transmission. STIs differ from other modes of transmission in that they are generally thought to cause chronic low level infections, which do not result in rapid septicaemia and increased host mortality associated with acute immune challenges (Lockhart et al. 1996). The lower initial inoculums in our STI treatment is evidenced by the proportion of flies that exhibit fungal growth on female cadavers (5-25% for STIs and 80-95% for DTIs; VL Hunt, unpublished data), and increased grooming activities we observed in the DTI treatment, which efficiently reduced pathogen load (this study; Phillis et al. 1993). Consistent with the differences in pathogen dose between the two infection treatments, we found that sexually transmitted *Metarhizium* infections cause weak, though significant, fitness costs for females, and

that the expression of *TotM*, but not *Dif*, ameliorates the survival costs associated with STIs. In contrast, we found that direct topical *Metarhizium* infections cause substantial fitness costs for females, and that the expression of *Dif*, but not *TotM*, helps ameliorate those survival costs. Taken together, these findings show fruit flies have a specific mechanism for immunity against low-dose STIs and against high-dose DTIs, even for the same pathogen.

It is important to acknowledge that though we have established a role for *TotM* in immunity against low-dose STIs, we do not know whether *TotM* confers immunity against STIs *per se*, or to low-dose infections more generally. We cannot dismiss the possibility that high fungal doses overwhelm the fine-tuned protective effects provided by *TotM* or that low fungal doses mask the susceptibility of the *Dif* knockdown. Similarly, the choice of diet could confound our results, as the fecundity benefits of *TotM* and *TotC* expression might have resulted from the *ad libitum* access to dietary yeast in this study (McKean et al. 2008). Another potential problem is that genetic constructs such the Act5c driver and UAS element may have pleiotropic effects on the life history of the fly, which could confound direct comparisons with the knockdown genotype. However, these problems are unlikely to influence our interpretations. The response to topical fungal infection in our *Dif* knockdowns was similar that of the classic *Dif* knockout mutant (Rutschmann et al. 2000). Because our experiments were conducted under the same diet and because our analysis included normalizations to genotype and underlying frailty, we can confidently attribute the survival reduction in *TotM* knockdown to the effect of gene expression rather than potential confounding factors such as diet, genetic pleiotropy, or the general frailty of immune gene knockdown lines (Le Bourg 2011). Regardless of how they confer immunity, our findings provide clear evidence that *TotM* and *Dif* are specific for different fungal transmissions, and that their expression has different life history consequences for the host.

It is important to stress that we are not arguing that *M. robertsii* is predominantly transmitted sexually or claiming that it is transmitted internally during copulation. Given the proclivity of *Metarhizium* for topical transmission, we would expect there to be some non-sexual transmission, even in our STI treatments. *Drosophila* tend to aggregate on food sources, which could have increased contacts and fungal transmission in the present study (Lihoreau & Simpson 2012). However, non-sexual transmission is not likely to be substantial enough to change our

interpretation of the data. First, males had been given 24 hours for grooming and were subsequently placed in fresh vials, which reduced the risk of females indirectly picking up dislodged spores. Second, we found that females who mated with infected males were more likely to be infected than those that did not. And, finally, in independent experiments, infection success was substantially lower in same-sex transmission trials than in trials involving sexual transmission (21% vs. 3%). Thus, although we documented that the fungus can be transmitted non-sexually, sexual transmission is primarily responsible for the observed infections in our STI treatments.

2.4.3 The cost of immune expression

Though many studies document costs of immunity (Moret 2000; Schmid-Hempel 2003; McKean et al. 2008), the molecular and physiological mechanisms which underlie these costs are often poorly understood (Sheldon & Verhulst 1996). We found that under uninfected control conditions *Dif* is generally deleterious in the absence of infections. The expression of *Dif* entails both significant survival and fecundity costs, which is also supported by a previous study of *Dif* knockout mutant (Le Bourg 2011). The costs of *Dif* expression are likely to arise from its control of AMP induction (Rutschmann et al. 2000), though *Dif* may also function in other non-immunity related processes (Lemaitre et al. 1996). These strong fitness costs could help to explain why *Dif* only appears to be modestly induced by direct topical fungal infections (De Gregorio et al. 2001), and why it was not upregulated in females in response to male courtship songs (at least in their heads) (Immonen & Ritchie 2012).

In contrast, our findings for the *Turandot* genes are only partially consistent with the predicted costs of immune gene expression. We found that *TotM* has an antagonistic pleiotropic influence on the life history of the fly: though it is costly for survival, expression of *TotM* also substantially enhances female fecundity. In addition, while there was no evidence that *TotC* conferred immunity against *Metarhizium*, it did not contribute to survival cost and even enhanced female fecundity. However, unlike *Dif*, there is evidence that *TotC* and *TotM* play additional roles in reproduction. They are upregulated in response to exposure to male accessory gland proteins (Lawniczak & Begun 2004; McGraw et al. 2004; Mack et al. 2006; Domanitskaya et al. 2007; McGraw et al. 2008). *TotM* could act to mediate

the trade-off between late-age survival and early-age reproduction, a key component of fitness in populations with fluctuating growth rates (N K Priest et al. 2008). Thus, though we cannot easily tease apart the costs of expression from the additional roles played by *TotM*, the fact that its expression induces survival costs indicates that *TotM* has a long-term detrimental effect, which is an important facet of the explanation for why it is not constitutively expressed. Interestingly, *TotM* and *TotC* appear to evolve more rapidly than *Dif* (Obbard et al. 2009), suggesting that they have experienced divergent or relaxed selection, perhaps as a consequence of their lower cost of expression (Stearns 1992; Schmid-Hempel 2003; McKean et al. 2008).

2.4.4 Mating and immune anticipation in insects

Mating is frequently associated with heightened risk of contracting both 'pure' STIs and other opportunistic infections (Crudgington & Siva-Jothy 2000; Stutt & Siva-Jothy 2001; Knell & Webberley 2004; Kamimura 2007). Such threats could be countered by upregulating immunity-related genes post-mating (Lawniczak & Begun 2004; McGraw et al. 2004; Mack et al. 2006; Domanitskaya et al. 2007; McGraw et al. 2008). However, because of the full deployment of immune responses can often take a considerable amount of time (Haine et al. 2008a & 2008b), selection is expected to favour immune anticipation of mating (Siva-Jothy 2009). Though there have been few well-documented cases, immune anticipation is likely to be far more common than currently appreciated. Our study supports the hypothesis that female fruit flies can mitigate the risk of contracting sexually transmitted fungal infections during mating by pre-emptively upregulating *TotM* (Immonen & Ritchie 2012). More generally, there are many other biological scenarios associated with elevated disease risk for which we would expect immune anticipation to be advantageous, such as feeding (as has been documented in bed bugs, M.T.Siva-Jothy *et al.*, unpublished) and crowding of conspecifics (Barnes & Siva-Jothy 2000; Wilson et al. 2001; Bailey et al. 2010). A particularly tantalising possibility is that the control of many immune genes, including *TotM* (McDonald & Rosbach 2001), by circadian clock genes might reflect 'anticipation' of predictable fluctuations of disease risk over the course of 24 hours. Thus, the courtship-induced, pre-emptive upregulation of *TotM* might be representative of a general pattern of immune anticipation in insects, underlining the intimate link between brain, behaviour and immunity (Ader & Cohen 1993; Maier et al. 1994).

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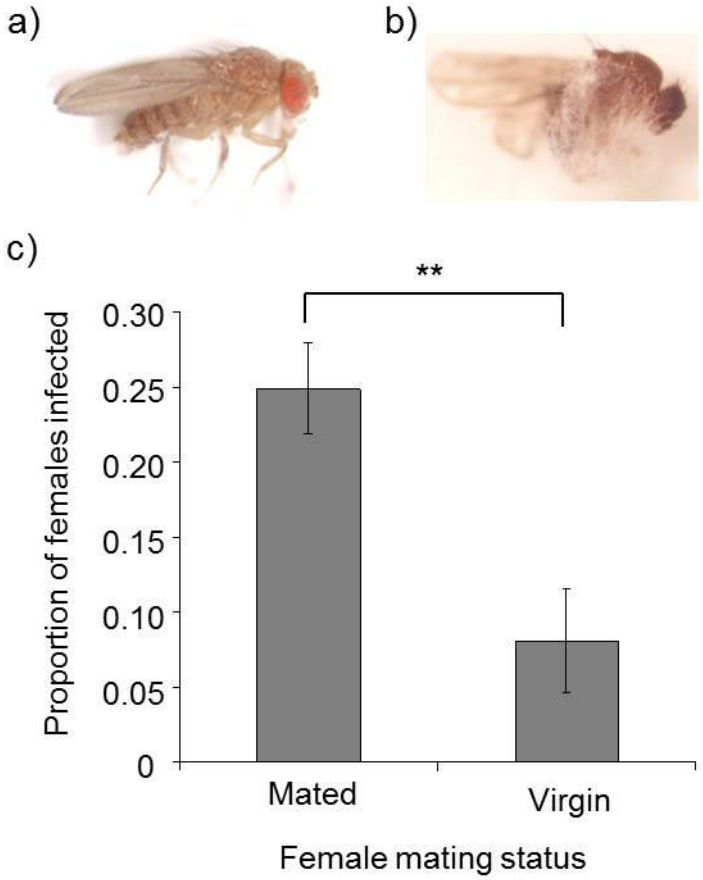

Figure 2.1 *Metarhizium robertsii* can be horizontally transmitted in *Drosophila melanogaster* as a result of mating. a), a female *Drosophila* covered in *Metarhizium* conidia immediately after direct topical infection; b), growing hyphae of *Metarhizium* emerging from infected fly cadaver; c), when kept with a *Metarhizium*-inoculated male, virgin females that mated were much more likely to acquire conidia than those that remained virgin.

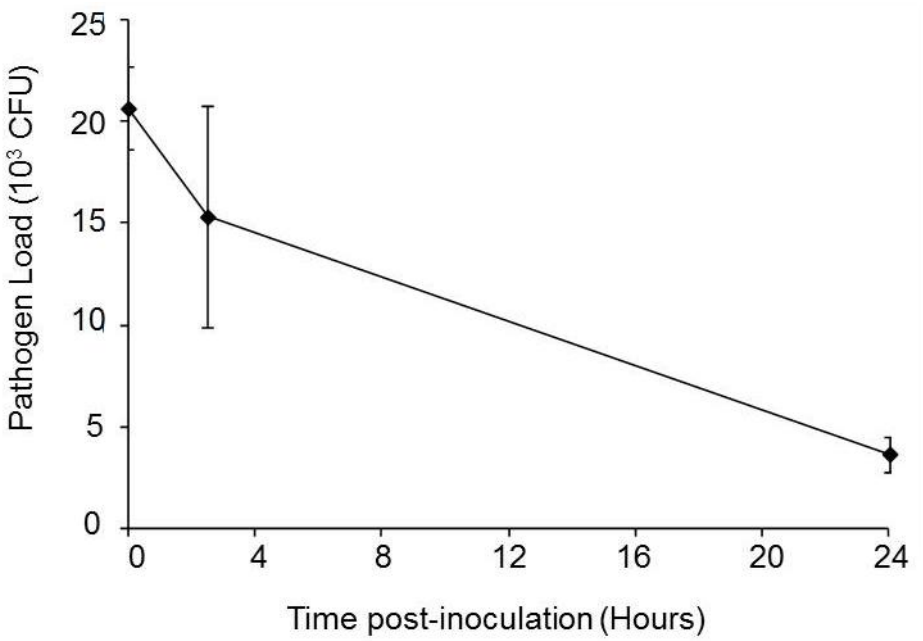

Figure 2.2 Fungal conidia load declines rapidly in the first 24 hours post inoculation (n=3 at each time point).

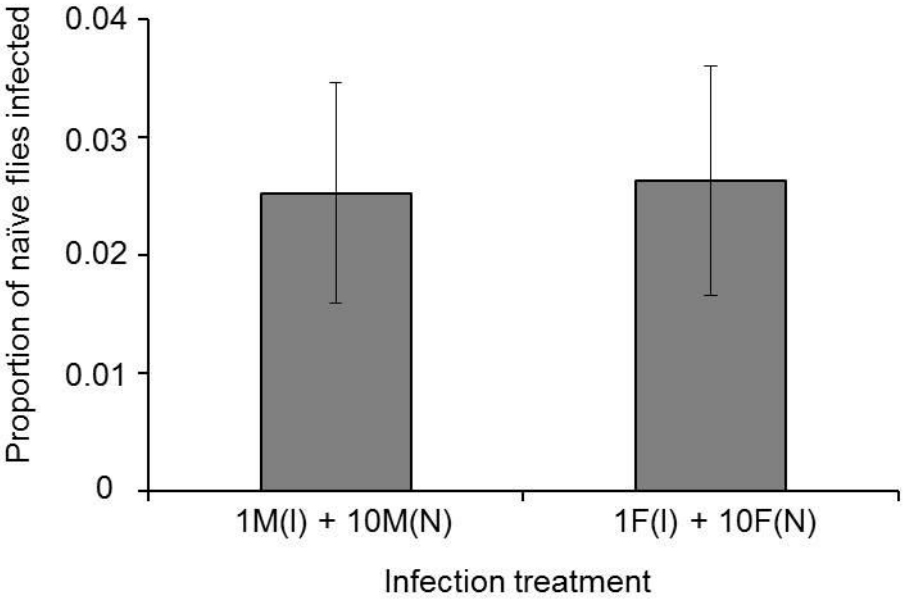

Figure 2.3 Non-sexual transmission of *Metarhizium robertsii* between fruit flies. M(I), infected male; M(N), naïve male; F(I), infected female; F(N), naïve female. Sample size of naïve flies: 1M(I) + 10M(N), n=284; 1F(I) + 10F(N), n=273.

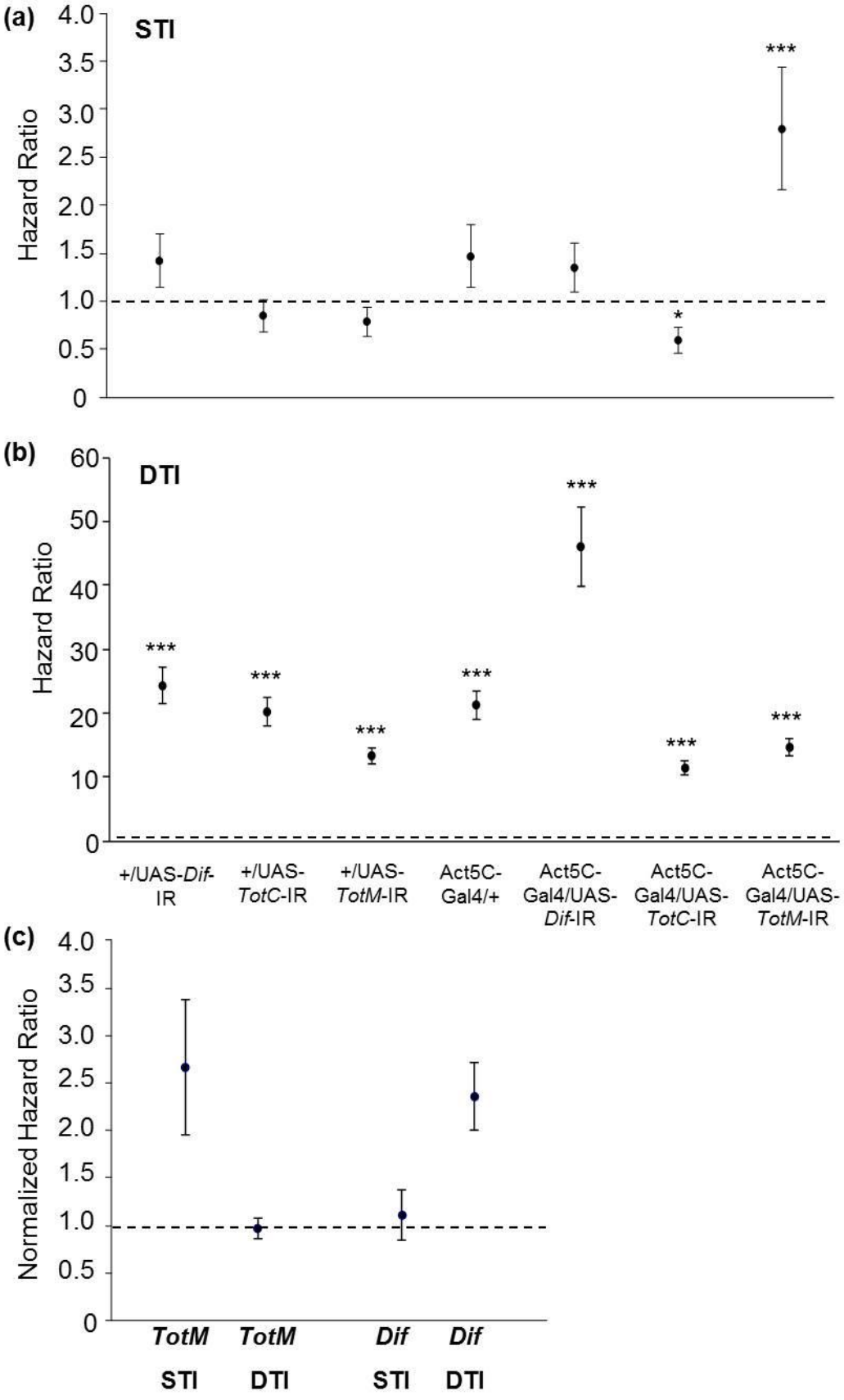
(a)
STI
Hazard Ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
***
*
(b)
DTI
Hazard Ratio
60
50
40
30
20
10
0
***
***
***
***
***
***
***
+/UAS-Dif-IR
+/UAS-TotC-IR
+/UAS-TotM-IR
Act5C-Gal4/+
Act5C-Gal4/UAS-Dif-IR
Act5C-Gal4/UAS-TotC-IR
Act5C-Gal4/UAS-TotM-IR
(c)
Normalized Hazard Ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
TotM STI
TotM DTI
Dif STI
Dif DTI

Figure 2.4 *TotM* is required for enhanced survival under sexually transmitted infection (STI), but not under direct topical infection (DTI). a), Cox proportional hazard ratios of STI relative to uninfected controls; b), Cox proportional hazard ratios of DTI relative to uninfected controls; c), relative effect of gene knockdown on infection susceptibility for *TotM*, *TotC* and *Dif* under both STI and DTI. Dotted lines indicate hazard ratio of 1, which indicate both infected and uninfected controls had the same risk of death. * indicates the level of statistical significance of hazard ratios (*, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

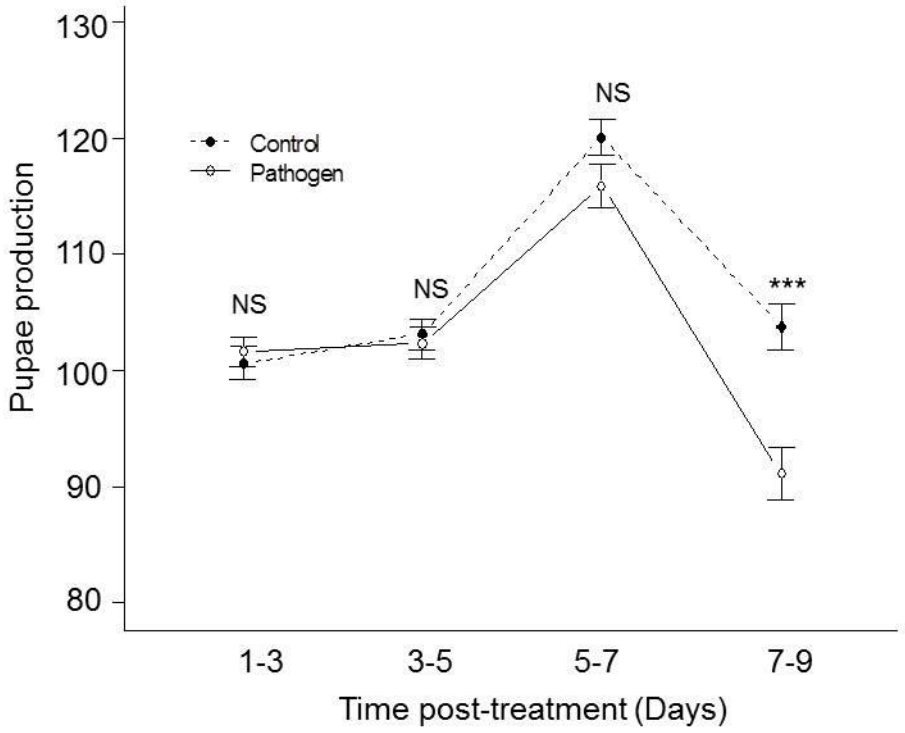

Figure 2.5 STIs cause fecundity loss for all genotypes. * indicates level of statistical significance (*, p< 0.05; **, p < 0.01; ***, p < 0.001). Student's t-test was performed for contrast at each time point.

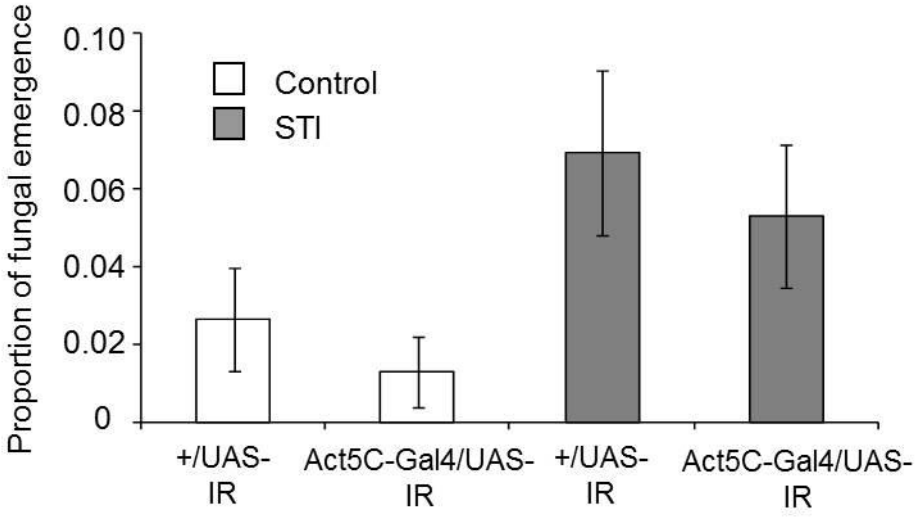

Figure 2.6 Exposure to fungus-infected male partners increase the likelihood of *Metarhizium*-like fungal growth on cadavers.

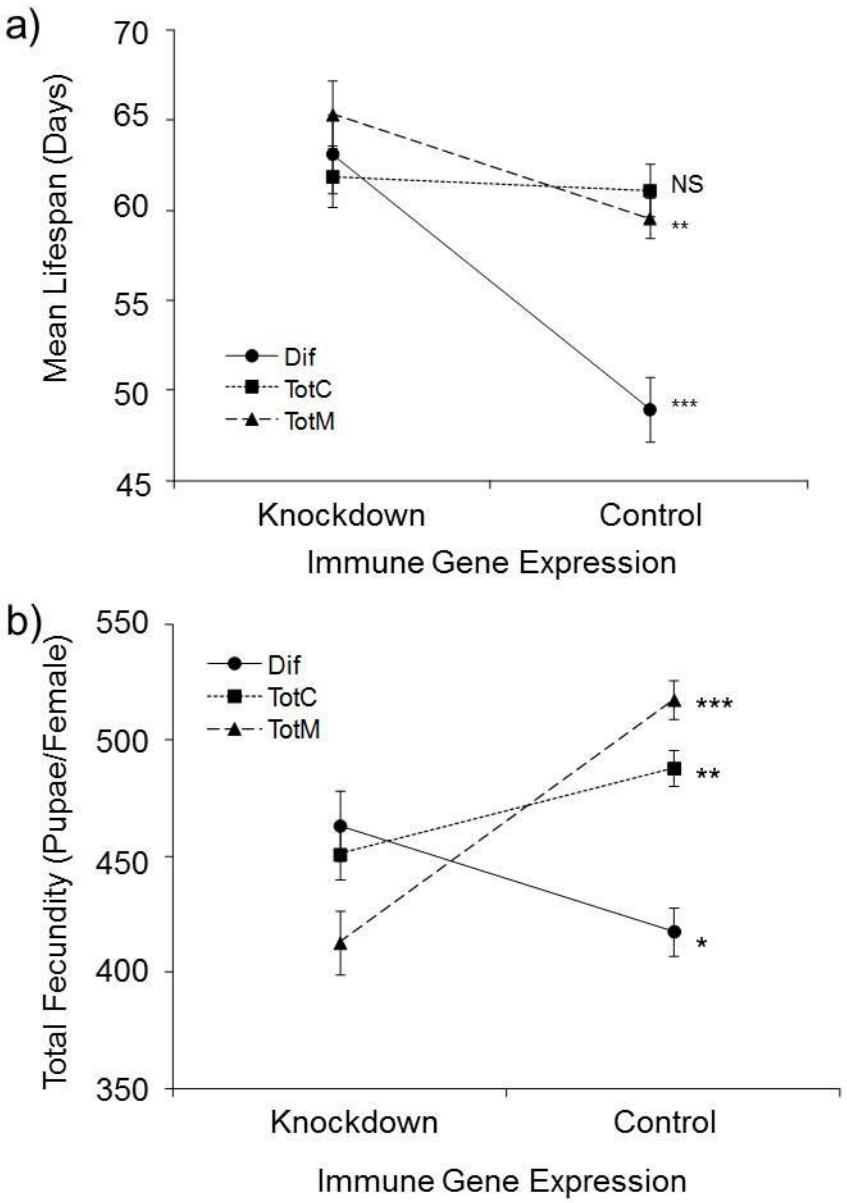

Figure 2.7 The costs of immune gene expression in the absence of infections. a), survival cost as measured by mean lifespan. b), fecundity cost as measured by total number of hatched pupae in the first 9 days post infection. * indicates level of statistical significance (*, $p< 0.05$; **, $p < 0.01$; ***, $p < 0.001$). For survival costs, statistical significance was based on Cox proportional hazard regression of the survival curves of knockdown and its combined control. For fecundity costs, statistical significance was based one-way ANOVAs.

Chapter 3 Parasite-induced cold seeking behaviour mediates an adaptive switch in life history

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Abstract

Animals must tailor their life history strategies to suit the prevailing conditions and respond to hazards in the environment. But, how they do this is largely unknown. Here we report that the fruit fly, *Drosophila melanogaster*, optimizes its life history by exploiting thermal variation. We find that uninfected control flies prefer warmer temperatures, which facilitates an *r*-like rapid propagation strategy. In contrast, fungus-infected flies prefer colder temperatures, which engenders a *K*-like fecundity reduction strategy that ultimately enhances lifetime reproductive success and resistance to the fungus. These findings help explain how life history trade-offs are mediated and how animals cope with infection in nature, which will be increasingly important given the recent emergence of fungal pathogens and global climate change.

Author contributions:
WZ, VLH & NKP conceived, designed and conducted the experiments, analysed data and prepared the manuscript. VLH designed, conducted and analysed the temperature preference assay. WZ designed and conducted the life history experiment, age-specific mortality analysis and pathogen growth assays. WZ, CDM, DTM & EMLD carried out the tolerance assay. AKC & NKP supervised the project.

* WZ & VLH contributed equally to the manuscript and are joint first authors.

3.1 Introduction

A central premise in evolutionary biology is that life history has evolved to maximise Darwinian fitness (Stearns 1992). Because they can experience radically different environments, even within a single generation, organisms must have the capacity to adjust their schedule of reproduction that best suit the prevailing environment i.e. adopt distinct life history strategies. Despite extensive research on the phenotypic plasticity of life history traits (Stearns & Koella 1986; Gotthard & Nylin 1995; Nylin & Gotthard 1998), how organisms shift their life history strategies in response to environmental change remains poorly understood.

Two of the most important factors shaping the life history of animals in nature are temperature (Huey & Berrigan 2001; Dillon et al. 2007) and parasitism (Minchella 1985; Michalakis & Hochberg 1994; Agnew et al. 2000; Hurd 2001). It is generally thought that animals seek out temperatures which maximize their fitness (referred to as the coadaptation hypothesis, Angilletta *et al.* 2006). Life history theory shows that early-life reproduction has a disproportionate contribution to the intrinsic rate of increase (r), an appropriate measure of Darwinian fitness in age-structured populations, when the population is expanding; but it is substantially less important in stable or declining populations, where late-age reproduction contributes more to fitness (Charlesworth 1994; Brommer 2000). Because the rates of development and early-life reproduction in poikilotherms generally tends to increase with ambient temperature up to their physiological optima (Taylor 1981; Huey et al. 1995; Angilletta et al. 2002; Dillon et al. 2007), we would expect healthy (i.e. expanding) populations to seek out relatively warm temperatures that maximise their intrinsic rate of increase. This prediction has been supported by empirical studies in fruit flies (Martin & Huey 2008) and warm-seeking strains of nematodes (Anderson *et al.* 2011).

Exposure to pathogens also stimulates shifts in animal life history. Theory generally predicts that parasitized hosts should exhibit fecundity compensation – an increase in current reproductive effort at the expense of reduced lifespan and late-age reproduction (Hochberg et al. 1992; Forbes 1993; Perrin et al. 1996). Another possibility is that infected animals could enhance their Darwinian fitness by fecundity reduction – a decrease in current reproductive effort that ultimately improves survival and lifetime reproductive success (LRS) (Hurd 2001). Such shifts

in life history in response to parasitism are predicted to occur because there are inherent trade-offs between reproduction and immune function e.g. the diversion of resources from reproduction to mounting costly immune defences (Sheldon & Verhulst 1996; Schmid-Hempel 2003). However, because parasites can also actively manipulate the allocation of host resources (Webb & Hurd 1999; Hurd 2003), it is often unclear whether documented shifts in life history represent adaptive changes in the strategy of the host, manipulation by parasites or unselected by-products of the infection process (Agnew et al. 2000; Hurd 2001).

Studies of parasite-induced changes in thermoregulatory behaviour could reveal how animals adjust their life history strategy in response to changes in their environment. Infected poikilotherms exhibit both warm-seeking (behavioural fever; Watson 1993; Adamo 1998; Elliot *et al.* 2002) and cold-seeking behaviours (behavioural anapyrexia;, Müller & Schmid-Hempel 1993; Zbikowska & Cichy 2012). Because the optimal temperature for *r* is generally higher than the optimal temperature for LRS across diverse poikilotherms (Huey & Berrigan 2001), we would expect behavioural fever to enhance *r* and facilitate fecundity compensation, whereas behavioural anapyrexia should enhance LRS and facilitate fecundity reduction. We would also expect the themoregulatory behaviour to enhance the host's ability to directly supress parasite growth and total parasite burden (i.e. resistance), or improve its ability to mitigate the detrimental impact of infection without direcly reducing parasite growth (i.e. tolerance), or a combination of the two (Schneider & Ayres 2008; Baucom & de Roode 2011; Medzhitov et al. 2012).

Previous studies of parasite-induced changes in thermoregulatory behaviour have focussed on their impact on the survival and immune function of infected hosts (Müller & Schmid-Hempel 1993; Watson 1993; Adamo 1998; Elliot et al. 2002; Zbikowska & Cichy 2012); however, little attention has been placed on the impact of temperature on reproductive strategy. To address the question of adaptive significance, we need a comprehensive approach, which not only directly measures the consequences of temperature preference on the reproductive output of infected hosts (in terms of both *r* and LRS), but also of naive control animals and sham-treated animals exposed to inactive pathogens. Here we employ the fruit fly, *Drosophila melanogaster*, as the host for the entomopathogenic fungus, *Metarhizium robertsii*, to examine the life history and immune consequences of parasite-induced changes in thermoregulatory behaviour. This system is ideal for this problem

because 1) *D. melanogaster* is a model species for the study of life history (Prasad & Joshi 2003), temperature preference (Dillon et al. 2009), innate immunity (Lemaitre & Hoffmann 2007), resistance and tolerance (Ayres & Schneider 2009), and the interactions between temperature and immunity (Lazzaro et al. 2008; Linder et al. 2008); but the thermoregulatory behaviour of fruit flies in response to infection is unknown. 2) *M. robertsii* is a common insect pathogen that has previously been shown to induce behavioural fever in locusts (Elliot et al. 2002; Ouedraogo 2003) and drives reporductive and survival costs in fruit flies (Zhong et al. 2013). Taken together, our study shows that thermoregulatory behaviour is a key mechanism employed by poikilotherms to adaptively tailor their life history strategy to the prevailing environment.

3.2 Materials and methods

3.2.1 Culture maintenance and infection treatment

With the exception of temperature preference tests, which also used the Canton-S and Tempe-T strains of *D. melanogaster*, all experiments used the Oregon-R strain that had been cultured at 25°C, 40% RH and 12:12 light/dark cycle on standard Nipagin-infused (an antifungal agent) oatmeal-molasses-agar media supplemented with a single grain of live baker's yeast. We cultured *M. robertsii* (isolate ARSEF 2575, previously classified as *M. anisopliae* strain ME1) at 28 °C in continuous light on one quarter strength Sabouraud Dextrose Agar with additional yeast extract (SDAy) and collected spores after 7-14 days. We infected flies by gently shaking cohorts of 10-15 flies in a 250ml conical flask containing 900μg of live spores for 10 seconds. Inoculated flies were sequentially transferred to fresh vials containing standard media to minimise the transfer of excess spores to the experimental vials. Control and heat-killed pathogen treatments were handled identically, except that the flask was empty or filled with 900μg of autoclaved (121 °C, 15 min) spores, respectively.

3.2.2 Temperature preference of Drosophila

We measured temperature preference of flies using on a purpose-built apparatus (**Figure 3.1a**, modified from Sayeed & Benzer 1996). Four escape-proof experimental lanes were created along the length of the apparatus with a perspex lid

and the application of the insect deterrent Fluon® to the inner walls encouraged flies to stay on the surface of the aluminium block. A piece of white paper placed on the aluminium block and marked into 10 equal sections was used to identify the position of flies across the gradient. A k-type thermocouple (Omega Engineering) was used to measure the mid-point of each section across the apparatus, which confirmed a linear temperature gradient ranging from 16 to 32°C along the aluminium block (**Figure 3.1b**).

We transferred control or infected mixed sex flies without anaesthesia in groups of 10-15 flies into the apparatus at 24, 48 and 72 hours post-infection. Temperature preference of each fly was established by recording their locations along the temperature gradient after 30 minutes. All flies were measured only once and the apparatus was cleaned with 70% ethanol after each trial and the paper marking the 10 sections replaced. Temperature preferences were assessed between 11:00 and 14:00 with a minimum of 4 trials for each pathogen treatment/time point. We also assessed the distribution of flies in the absence of thermal gradient at room temperature (**Figure 3.1c**).

3.2.3 Effect of temperature on host survival and age-specific mortality

We treated 3-4 day old mixed sex adult flies with live, heat-killed or control pathogen treatment and kept in population cages at 22 or 25°C until all animals had died (11 × 15 cm; n = 50 cages at approximately 40 flies/cage). The cages were provided fresh fly media vials daily and dead flies were removed and recorded. We also confirmed the cause of death by random sampling of cadavers and examining them for signs of *Metarhizium*-like fungal growth. Sampled cadavers were surface sterilized (brief immersion in 1% bleach, 70% ethanol and sterile water) and placed on filter paper moistened with sterile water in sealed Petri dishes. The resulting plates were kept at 28°C for up to 10 days to check for external fungal growth under a dissection microscope.

3.2.4 Life history consequence of temperature preference and infection

Two day old adult virgin females were allowed to mate with males for 24 hours at 25°C in groups of 20 flies. Males were then discarded and females were maintained for a further 24 hours at 25°C. We treated females (adult age 4 day; n = 259) with one of three pathogen treatment (control, live and heat-killed fungus) and transferred

them into individual vials containing fresh media in 10 randomised blocks at 22 or 25°C. Every 2 days post-infection, we counted the number of eggs laid by each female and replaced the food media with fresh vials until the fly died. The number of adult deaths was also recorded after each egg collection. We incubated vials containing eggs incubated at 25°C and examined them again after 14 days to record the number of eclosed pupae.

3.2.5 Effects of temperature on fungal growth, host resistance and tolerance

Because host temperature preference could function as a mechanism of resistance (i.e. direct suppression of the growth of parasites) we assessed the growth rates of *M. robertsii* both on artificial media and live hosts. For *in vitro* growth, we placed 4mm non-sporulating mycelial plugs in the centre of SDAy plates at 22, 25 and 28 °C in continuous darkness (n=30; modified from Ouedraogo *et al.* 1997). We then measured the diameter of fungal mycelium daily along two perpendicular axes drawn on the petri dish for a total of 8 days. We estimated *in vivo* growth of rate *M. robertsii* from periodic sampling of infected host parasite load. Mixed sex fruit flies were first inoculated with 20-25 mg of *M. robertsii* according to the infection protocol (11 × 15 cm; approximately 350 flies/cage). Treated flies were then placed at five temperatures ranging from 18 to 28°C over a period of 17 days (n = 30 cages). We randomly sampled pairs of surviving flies on day 3, 5 (28°C treatment only), 7, 10, 14 and 17 post-inoculation for all temperature treatments (n=147 flies). Sampled flies were individually surface sterilised (brief immersion in 1% bleach, 70% ethanol and sterile water) and homogenised in a buffer of 0.04% Tween®80. The homogenates were spread on fresh SDAy plates and incubated at 28°C for three days after which *M. robertsii*-like colony forming units (CFUs) were identified by spore morphology using a microscope and counted. In cases where the number of CFUs on the plate was too large to count (>700), parasite load was estimated by multiplying the mean CFUs within $1cm^2$ sample squares by the number of squares.

We defined host tolerance as the norm of reaction between parasite load and mortality risk (Simms 2000; Baucom & de Roode 2011). For host mortality rates, we recorded the daily number of deaths from the same fly populations that we sampled flies for estimating parasite load.

3.2.6 Statistical analysis

All statistical analyses were carried out with SPSS version 13.0 and R version 2.11.1 (R Development Core Team 2010). Planned treatment contrasts were used to assess significance between treatments within full statistical models.

We analysed temperature preference with chi-squared tests by pooling flies from all replicate trials and dividing them into three temperature categories along the temperature gradient (cold, medium and warm). For host survival and mortality, we first fitted Cox proportional hazard regressions with parasite, temperature and parasite × temperature using female survival data (models fitted using males gave qualitatively similar results). We then converted the survival data into natural-log transformed mortality rates before fitting age-specific mortality models (Pletcher 1999; Pletcher et al. 2000). The parameters of the age-specific mortality models were estimated using minimised sums of squares and maximum likelihood methods in the R package 'Survomatic' (version 1.4.0.0). We combined the sexes for model fitting as we did not did not find any sex differences in mortality patterns (data not shown). Individual Gompertz and the more complex logistic models were fitted to each pathogen/temperature treatment combination. Gompertz model describes the classic pattern where mortality increases exponentially with age and has the hazard function ae^{bt} where t is the age at death, a is the morality intercept (background or age-independent mortality), and b is the rate of increase in mortality (rate of ageing). Logistic model modifies Gompertz model by adding the s parameter, the rate of deceleration of mortality at older ages (mortality levelling-off) and is described by the hazard function $ae^{bx}[1 + (a\ s/b)(e^{bx} - 1)]^{-1}$. Log-likelihood ratio tests were used to assess the significance of the differences between estimated parameter values for all treatments.

We assessed the effect of temperature and infection on two measures of reproductive fitness (LRS and r). LRS was measured as the total number of eclosed pupae over the entire lifetime for each female. r was estimated for each treatment combination within each block using the number of eclosed pupae produced at each collection interval (n=30). We obtained estimates of r by solving numerically the discrete form of the Euler-Lotka equation: $1 = \Sigma\ e^{-rx} l_x m_x$, where x is the age class, l_x is the probability of surviving from age class x to age $x+1$, and m_x is the expected number of offspring for a female in age class x (Charlesworth 1994). We then performed separate ANOVAs on LRS and r that included parasite, temperature, and parasite × temperature. To examine the effects of temperature and infection on age-

specific fecundity, we fitted separate linear mixed effects models to eggs and pupae data. The full models included eggs or pupae as the response variable; age, pathogen, temperature and all their interaction terms as fixed effects; Individual females were treated as a random effect. We excluded fecundity data in the first two-day interval (day 2-4 post-inoculation) from analysis of age-specific fecundity to improve the model fit. Repeating the analysis with the first interval yielded similar results (data not shown).

For host resistance, we assessed the effect of host temperature preference on the growth rates of *M. robertsii* both on artificial media and live hosts. For *in vitro* fungal growth, one-way ANOVA was performed on mean daily growth rates on replicate media plates (mm/day). For growth rate within live hosts, we performed 1) linear regression, with a quadratic term, on the estimated growth rates of individual replicate cages up to peak CFU counts (n=30; ln(CFU)/day); 2) linear regression on the time taken for each replicate cage population to reach peak CFU counts; and 3) mixed effects model on log-CFU counts over time, with temperature and time as fixed effects and cage as a random effect.

For host tolerance, we used mixed effects models to assess the relationship between parasite load and host mortality across five temperatures. Significant temperature effect on the slope of the correlation between parasite load and host mortality (parasite load × temperature interaction) would indicate changes in levels of host tolerance i.e. the ability of the host to maintain low mortality despite increasing parasite load. At each time point, CFU counts obtained from the same cage were averaged and log-transformed. Age-specific mortalities were estimated by calculating the proportion of flies dying on the day of live fly sampling and two days preceding it (i.e. within 72 hours of live fly sampling). The full model included natural log-transformed age-specific mortality as response variable; parasite load (ln(CFU)), temperature, parasite load × temperature as fixed effects; time (number of days post-inoculation) was added as a covariate to account for non-independence of mortality rate estimates over time. Individual cage was fitted as a random effect. An autoregressive error structure was fitted using *corAR1()* function in R package 'nlme' (following Pollitt *et al.* 2012).

3.3 Results

3.3.1 Temperature preference of Drosophila

When placed on a linear temperature gradient, control flies typically preferred ~25^{o}C (**Figure 3.2a-c**), but switched their preference to colder temperatures (~22^{o}C) within 24 hours after topical inoculation with live *M. robertsii* spores (**Figure 3.3**). This behavioural anapyrexia persisted until at least 72 hours post-inoculation (Live pathogen vs No pathogen control: $\chi^2 > 15.2$, $p < 0.002$ at all time points) and was consistent across multiple laboratory strains of *D. melanogaster* (**Figure 3.4a-c**). Interestingly, flies treated with heat-killed spores also exhibited behavioural anapyrexia at 24 and 72 hours post-inoculation, though to a lesser degree than live pathogen treated flies (Heat-killed pathogen vs No pathogen: 24hours, $\chi^2 = 8.22$, $p=0.04$; 72 hours, $\chi^2 = 12.0$, $p=0.007$).

3.3.2 Effect of temperature on host survival and age-specific mortality

We found that the colder temperature preferred by *Metarhizium*-infected flies extended survival post-infection, but control flies preferred warmer temperatures that elevated their risk of death. As expected, independent survival experiments confirmed that exposure to live fungal spores reduced the survival of flies (Parasite, $\chi^2_2 = 925$, $p< 0.0001$), and that residing at 22^{o}C conferred survival benefits to infected flies relative to staying at 25^{o}C (Temperature, $\chi^2_1 = 214$, $p< 0.0001$; **Figure 3.5a,b**). However, infected flies do not benefit proportionally more from colder temperature than uninfected control or heat-killed fungus treated flies (Parasite × Temperature, $\chi^2_2 = 3.11$, $p= 0.21$). The same pattern was confirmed by age-specific analysis of mortality rates. In contrast to the control and heat-killed fungus treatments, which fitted the simple Gompertz mortality trajectories (**Figure 3.6a,b**), the live pathogen treatment significantly increased the age-dependent mortality rate and caused a prolonged period of mortality "levelling off", justifying more complex logistic mortality models ($\chi^2_1 > 2.7$, $p< 0.05$ for live pathogen treatment at all temperatures; **Figure 3.6c**). However, colder temperature was only associated with reduced background (age-independent) mortality, and this effect was found in all pathogen treatments ($\chi^2_1 > 7.8$, $p< 0.001$ for all treatments).

3.3.3 Life history consequence of temperature preference and infection

We found that parasite-induced behavioural anapyrexia results in an adaptive switch in the life history of female fruit flies from a strategy that favours early-age reproduction and *r* to one that favours later age reproduction and LRS. Relative to 25^{o}C, flies residing at 22^{o}C generally had lower intrinsic rate of increase ($F_{1,24}$ = 19.4, p <0.001; **Figure 3.7a**), but higher lifetime reproductive success (Temperature, $F_{1,227}$ = 4.0, p =0.047; **Figure 3.7b; Table 3.1**), though the effect on LRS was only significant in the live pathogen treatment (*a priori* contrast, t=2.3, p=0.021). Surprisingly, although exposure to live *Metarhizium* spores greatly reduced total egg production of flies ($F_{2,227}$ = 132, p < 0.0001; **Table 3.1**), we found that overall pathogen treatment did not significantly reduce either LRS or *r* of infected flies (LRS, $F_{2,227}$ = 1.08, p = 0.34; *r*, $F_{2,24}$ = 2.34, p =0.18).

Age-specific analysis of female reproductive output (eggs) further revealed that temperature and infection had complex effects on the pattern of reproduction in the fruit fly (Age × Temperature × Parasite: $F_{2,4923}$ = 15.5, p < 0.0001; **Figure 3.8a-c; Table 3.2**). From the peak at day 2-4 post-inoculation, egg production declined rapidly with age in all treatments (Age, $F_{1,4923}$ = 218.3, p < 0.0001). Flies residing at 25^{o}C had significantly higher early-age fecundity at the expense of much lower late-age fecundity than those at 22^{o}C (Age × Temperature, $F_{1,4923}$ = 77.3, p < 0.0001). However, while the fecundity patterns of infected flies were initially similar to the control treatments at the two temperatures (0-2 days post-inoculation), as the infection progressed (after 4-6 days post-inoculation), the decline in fecundity was much slower for infected animals residing at 22^{o}C than those at 25^{o}C (Live pathogen: Age × Temperature, $F_{1,237}$ = 9.8, p = 0.002; **Figure 3.8c; Table 3.3**).

3.3.4 Fungal growth, host resistance and tolerance

We found that the colder temperature preferred by *Metarhizium*-infected flies enhanced host resistance, but not tolerance to fungal infections. Colder temperatures reduced fungal growth rate both *in vitro* and *in vivo* ($F_{2,15}$ = 68.9, p< 0.0001; $F_{1,26}$ = 21.7, p< 0.0001; **Figure 3.9a,b**) and increased the time taken to reach peak parasite load in the live host ($F_{1,18}$ = 48.5, p <0.0001). Thus, cold-seeking behaviour is an effective mechanism of resistance against fungal infections. In contrast, we found no evidence that cold-seeking behaviour affected tolerance. Because the rise and subsequent fall in parasite load occurred more rapidly at warmer temperatures

(**Figure 3.10**), the parasite load at any particular temperature depended greatly on the time post-inoculation at which it was measured (Temperature × Age, $F_{4,103} = 3.40$, $p = 0.012$; **Table 3.4**). Using mixed effects regression models, we found that while both higher parasite load ($F_{1,98} = 4.57$, $p = 0.035$) and warmer temperatures ($F_{4,25} = 7.7$, $p = 0.026$) increased the host's risk of death, moving to the colder temperatures did not enhance the capacity of the host to mitigate the harmful effects of fungal infection on mortality (Parasite load × Temperature, $F_{4,98} = 0.96$, $p = 0.43$; **Figure 3.11**; **Table 3.5**).

3.4 Discussion

There has been extensive interest in understanding how organisms balance the allocation of resources to life history traits (Stearns 1992; Charlesworth 1994). However, the mechanisms by which organisms adjust their life history in response to environmental change are poorly understood. It is also unclear whether those changes always result in greater Darwinian fitness. We found that in addition to being an effective mechanism of immunity, parasite-induced thermoregulatory behaviour functions as a means for rapid and easily-reversible adjustment of host life history strategy. Our findings are important because they show that phenotypically plastic life history responses mediated by thermoregulatory behaviour can mitigate the cost of parasitism, and highlight that measuring classic fitness measures, LRS and *r*, within the same experiment can yield novel insights (Huey & Berrigan 2001; Anderson et al. 2011).

3.4.1 Parasite-induced thermoregulatory behaviour and host survival

'Non-immunological' mechanisms such as thermoregulatory behaviour are increasingly appreciated as critical components of an animal's defence against parasites (Thomas & Blanford 2003; Parker et al. 2011; de Roode & Lefèvre 2012). In contrast to the well-documented phenomenon of behavioural fever (Watson 1993; Adamo 1998; Elliot et al. 2002; Richards-Zawacki 2010), though consistent with other cases of behavioural anapyrexia (Müller & Schmid-Hempel 1993; Zbikowska & Cichy 2012), we found that *D. melanogaster* infected with the fungus *M. robertsii* preferred colder temperatures than uninfected control animals. This switch in temperature preference is likely to be driven by the host, rather than being a result of

parasite manipulation, since even flies treated with heat-killed fungus displayed increased preference for colder temperatures (**Figure 3.3**).

Though previous work has argued that behavioural fever and anapyrexia provide survival benefits for infected animals (Müller & Schmid-Hempel 1993; Adamo 1998; Elliot et al. 2002; Richards-Zawacki 2010), we find that survival benefits alone are not sufficient to explain why infected animals prefer colder temperatures. While cold-seeking behaviour indeed enhanced the survival of *Metarhizium*-infected fruit flies, uninfected control flies also received survival benefit by residing at colder temperatures. The survival benefit of colder temperature did not derive from reduced rates of ageing (in control animals) or physiological decline (in infected animals). Instead, lower temperature greatly reduced the background risk of death, but its influence was roughly equivalent within each pathogen treatment. Thus, if colder temperature provides universal survival benefits, why should uninfected flies prefer significantly warmer temperatures?

3.4.2 Life history consequences of temperature preference

Our results suggest that fruit flies exploit their thermal environment to adjust their life history strategies, the pattern of age-specific reproduction. Uninfected poikilothermic animals in expanding populations are expected to favour fast development and early-age reproduction, both of which are positively influenced by ambient temperature (Taylor 1981; Huey et al. 1995; Dillon et al. 2007), in order to maximise their intrinsic rate of increase (Charlesworth 1994; Brommer 2000). Previous studies using *D. melanogaster* indicate that adult flies have strong temperature preference at approximately 24-25^{o}C (Sayeed & Benzer 1996; Dillon et al. 2009), we confirmed this in uninfected control flies and found that they achieved higher intrinsic rate of increase at 25^{o}C than those kept at 22^{o}C. This is consistent with previous finding that in *Drosophila r* is maximised at 25^{o}C (Martin & Huey 2008). In contrast, we found that lifetime reproductive success was not significantly different at the two temperatures in uninfected control treatments. Together with the recent study in nematodes (Anderson et al. 2011), our results suggest that at least in some populations of poikilotherms, temperature preference might have evolved to maximise intrinsic rate of increase.

When exposed to parasites, hosts could adopt fecundity compensation or fecundity reduction strategies (Chadwick & Little 2005; Hurd 2001). We found that

when they had access to thermal variation, infected animals chose cooler temperatures, which reduced their early age fecundity and *r*, but enhanced late-age reproduction and LRS, a pattern suggestive of a fecundity reduction strategy. This result is consistent with the observation that the temperature that maximises *r* is often greater than that which maximises LRS in poikilotherms (Huey & Berrigan 2001). The adaptive values of fecundity compensation or reduction are likely to depend on the demography of the population. In particular, fecundity compensation might be maladaptive declining populations associated with parasite-rich environments (Charlesworth 1994; Brommer 2000).

3.4.3 Temperature preference and immune trade-offs

Temperature preference alters life history strategies by mediating the trade-off between reproduction and immunity. Although we know immunity has costs which result in trade-offs with other components of fitness (Sheldon & Verhulst 1996; Schmid-Hempel 2003), the mechanisms by which animals mediate these trade-offs to enhance fitness are poorly understood (Flatt *et al.* 2005). We found that fruit flies at warm temperatures achieved high early-age reproduction and *r* at the expense of resistance to fungal parasite; however, by moving to colder temperatures, they improved their antifungal resistance at a cost to *r*. Cold-seeking behaviour enhanced resistance against *Metarhizium* infections because colder temperature directly reduced germination success and vegetative growth of fungal spores (this study; Ouedraogo *et al.* 1997). Temperature could also have influenced the expression of host immune genes (Linder *et al.* 2008) and function of haemocytes (Ouedraogo 2003). Interestingly, we found that the colder temperature did not enhance tolerance to fungal infection; defined here as the reaction norm between parasite load and host mortality risk (Simms 2000; Baucom & de Roode 2011), which suggests that resistance and tolerance can vary independently (Ayres & Schneider 2008). Together, these results suggest that if we only conduct our experiments under a single thermal condition, we may not be able to accurately assess the costs and benefits of immunity (Moret & Schmid-Hempel 2004; Lazzaro & Little 2009).

3.4.4 Caveats

The principle caveat of these findings is that our design may have augmented the consequences of temperature on life history and immunity. We deliberately

controlled for the multitude of factors which influence *Drosophila* temperature preference such as rearing temperature and humidity (Good 1993; Krstevska & Hoffmann 1994; Dillon et al. 2009) by conducting all pathogen treatments simultaneously with uninfected controls under carefully controlled conditions. But by excluding potential confounding environmental factors we might limit the ecological relevance of what we have found for field situations (Cisarovsky *et al.* 2012). We also used lab-adapted strains of *Drosophila* strains which could have diverged from wild populations in their life history and temperature preference. In particular artificial culture methodologies could have inadvertently selected for increased early age fecundity (Sgro & Partridge 2000) and higher temperature preference (McDaniel et al. 1995) in uninfected control flies. However, these possibilities are unlikely to influence our conclusions as we identified consistent temperature preferences among lines which have been exposed to different forms of lab adaptation. Lastly, it might appear counter-intuitive that even though we found that live fungal infection greatly reduced total egg production of infected flies, this did not translate into significantly reduced *r* or LRS based on the number of eclosed pupae. This could be explained by the fact that 1) infection has weak effects on reproduction very early in life which also contributes the most to measurements of *r*; and 2) because mating opportunities were limited to a single 24 hour window at the start of the experiment and female *D. melanogaster* store relatively small numbers of sperm after a single mating (Lefevre & Jonsson 1962), sperm storage was likely to be depleted in uninfected control females resulting in fewer viable offspring.

Nevertheless, our findings suggest that the exploitation of thermal variation is an important mechanism for poikilotherm to tailor their life history strategies in changing environments. In particular, we hope that these results stimulate further experimental work that directly assess the importance of this mechanism in wild populations where poikilotherms are subject to fluctuating environmental temperatures and pathogenic exposure. It has recently been suggested that behavioural thermoregulation will be a key mechanism for poikilothermic animals to buffer the impacts of global climate change (Gvozdík 2012; Kearney et al. 2009). Moreover, given the recent global spread of fungal pathogens (Fisher *et al.* 2012), thermoregulatory behaviours are likely to play increasingly important roles in the defence against these threats.

3.5 References

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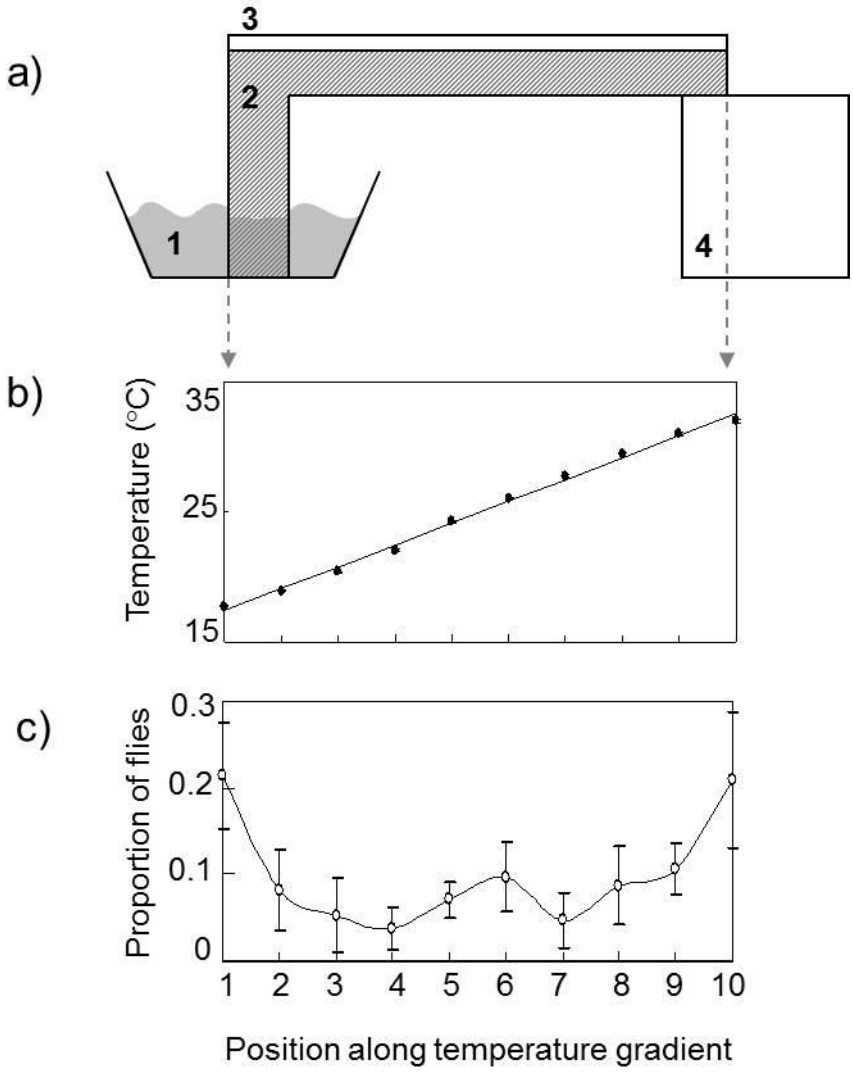

Figure 3.1 Apparatus for measuring temperature preference of *Drosophila*. a) The experimental apparatus used to measure temperature preference. It consisted of a cold water bath (1), an 'L' shape aluminum block (270 × 180 × 30 mm) (2), a perspex lid (5mm in height) (3) and a hot plate (4). b) Measurements at fixed positions along the gradient indicate a temperature range of 16 to 32°C. c) The distribution of Oregon-R flies at room temperature in the absence of a temperature gradient in the apparatus.

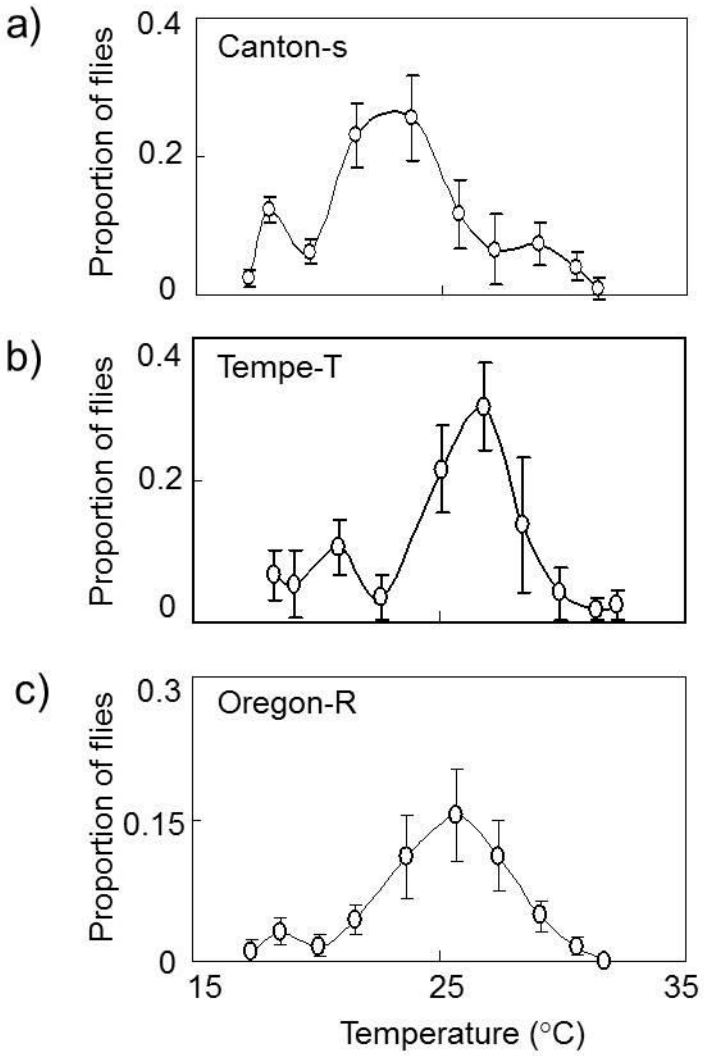

Figure 3.2 Temperature preference in three uninfected control laboratory strains of *Drosophila melanogaster*. a) Canton-S flies prefer mean temperature of 23.5 ±0.4°C (n = 77). b) Tempe-T flies prefer mean temperature of 25.2 ±0.6°C (n = 37). c) Oregon-R flies prefer mean temperature of 24.4 ±0.2°C (n = 220).

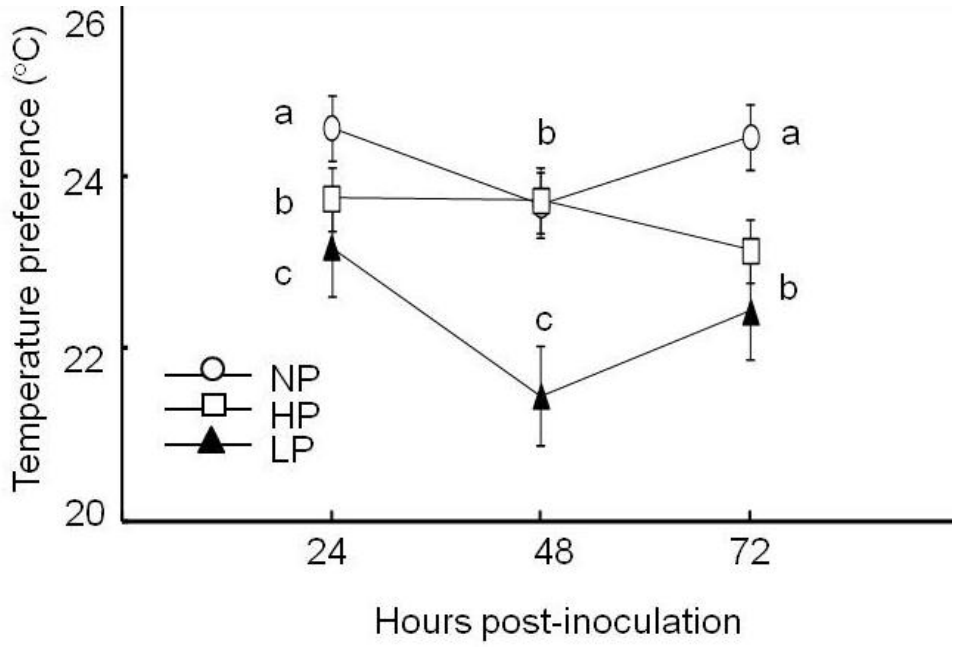

Figure 3.3 *Drosophila melanogaster* infected with *Metarhizium* prefer colder temperature relative to uninfected control animals. Flies were topically inoculated with live *Metarhizium robertsii* spores (LP, triangles), heat-killed *M. robertsii* spores (HP, squares) or no pathogen controls (NP, circles) and placed on a temperature gradient ranging from 16 to 32°C at three time points post inoculation. Within each time point (24, 48 and 72hours), different letters indicate significant difference at p = 0.05 (χ2 test).

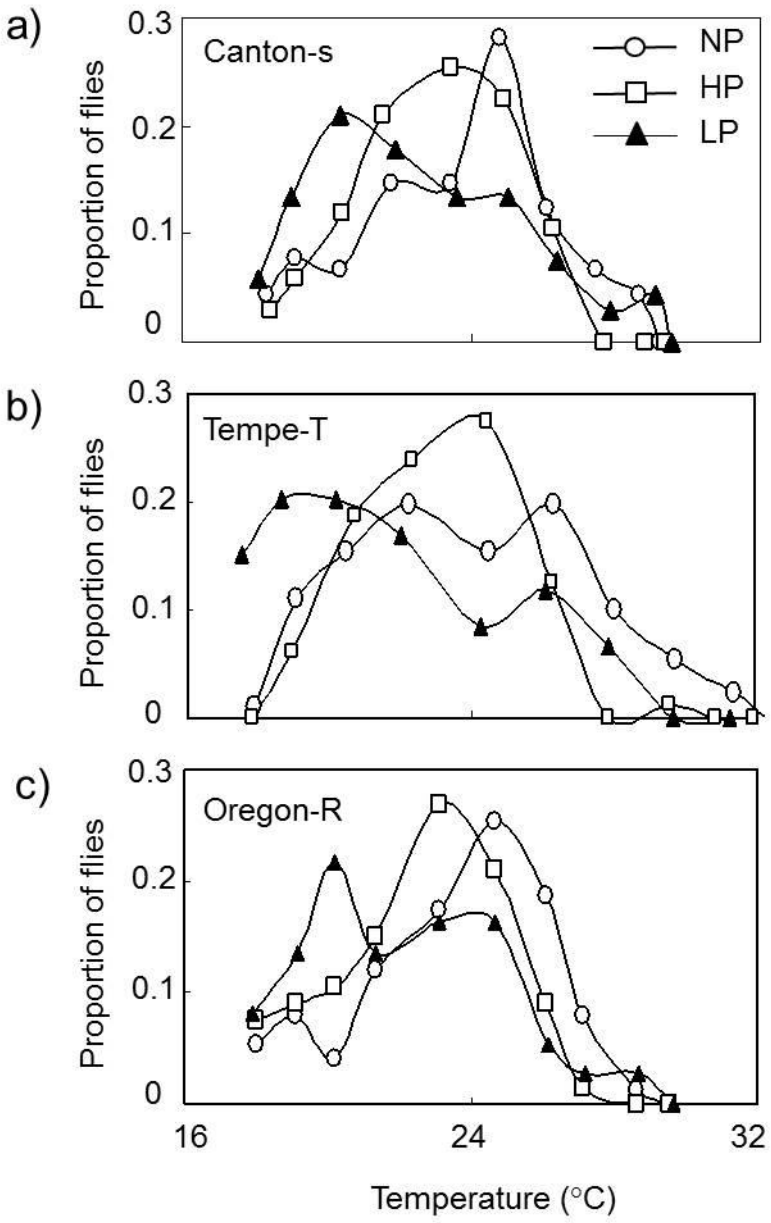

Figure 3.4 Temperature preference in *Drosophila melanogaster* exposed to live *Metarhizium* spores is shifted towards the cold. Temperature preference was assessed at 48 hours post-inoculation. a) Canton-S flies (n = 222). b) Tempe-T flies (n = 215). c) Oregon-R flies (n = 189).

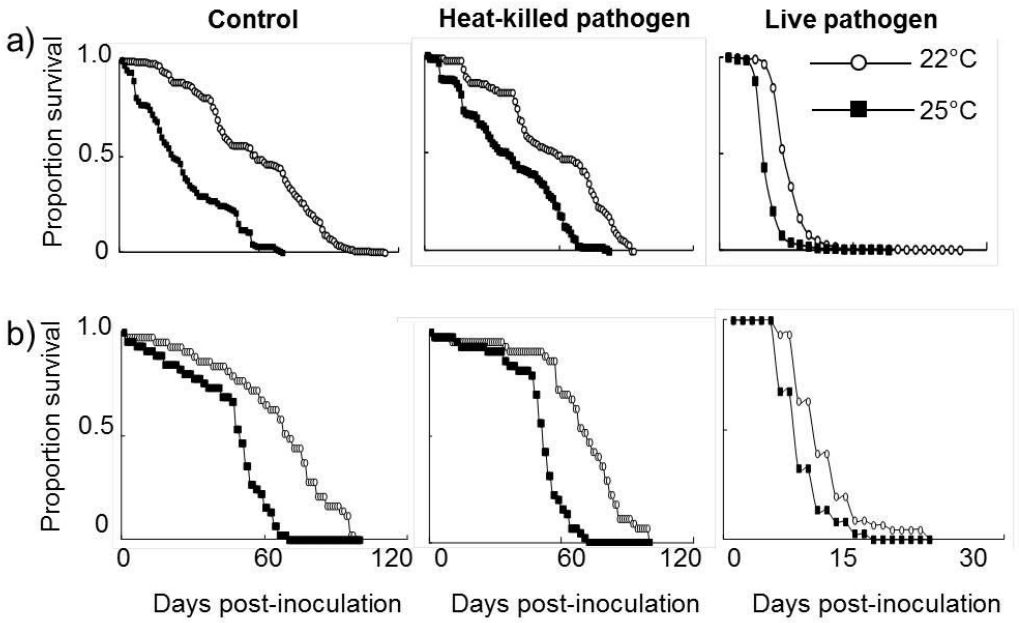

Figure 3.5 Cooler temperature enhances survival in all pathogen treatments across independent experiments. a) Survival curves for mixed sex fruit flies observed in large population cages used in the demographic experiment (n = 2088). b) Survival curves for female fruit flies observed in individual vials used in the life history assay (n=259).

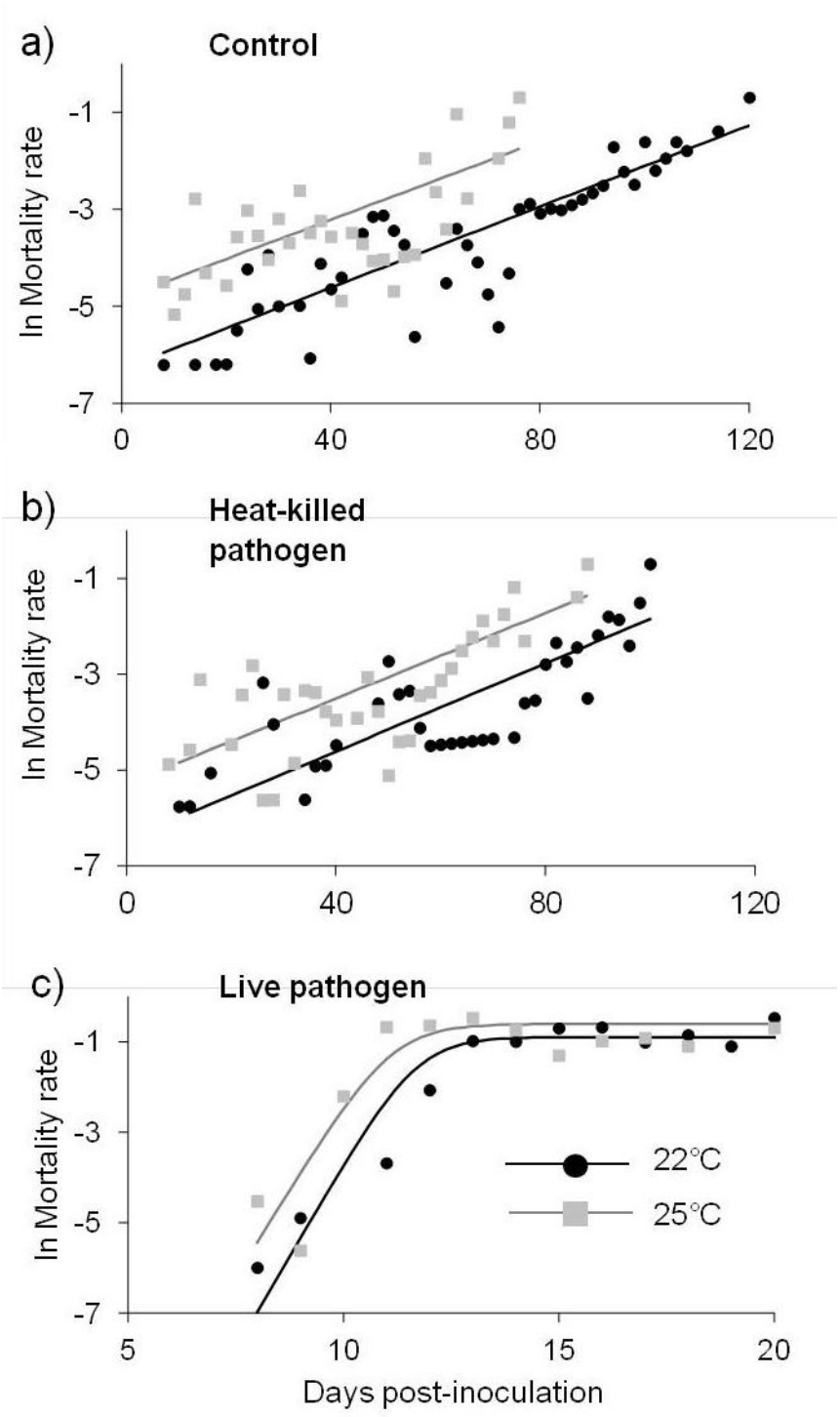

Figure 3.6 Temperature influences age-independent mortality while *Metarhizium* infection affects age-dependent mortality in *Drosophila.* a) Sham controls. b) Flies inoculated with heat-killed *M. robertsii* spores. c) Flies inoculated with *M. robertsii* spores. Data shown are natural log-transformed daily mortality rate. Fitted lines are Gompertz (a & b) and Logistic mortality models (c).

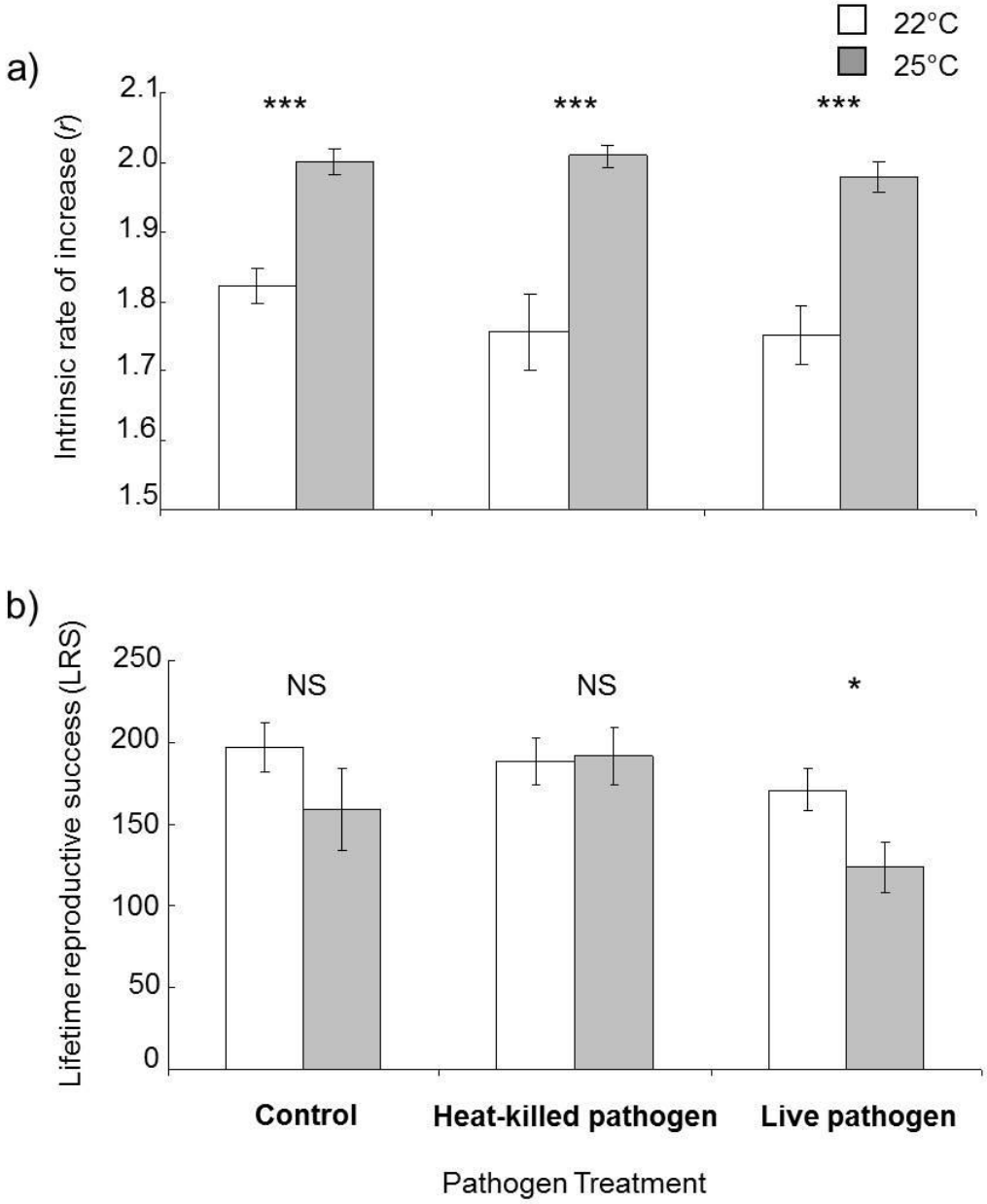

Figure 3.7 Fitness consequences of cold-seeking behaviour. a) Intrinsic rate of increase (*r*) across temperature and pathogen treatments. *r* was calculated using pupae produced at each collection interval for each block (n = 30). b) Lifetime reproductive success (LRS) across temperature and pathogen treatments. LRS was measured as the total number of eclosed pupae from the start of treatments until death for each female (n =244). All error bars are standard errors of the mean. Statistical significance was established by a priori treatment contrasts specified in ANOVA (**, $p < 0.01$; ***, $p<0.001$; NS, $p > 0.05$.)

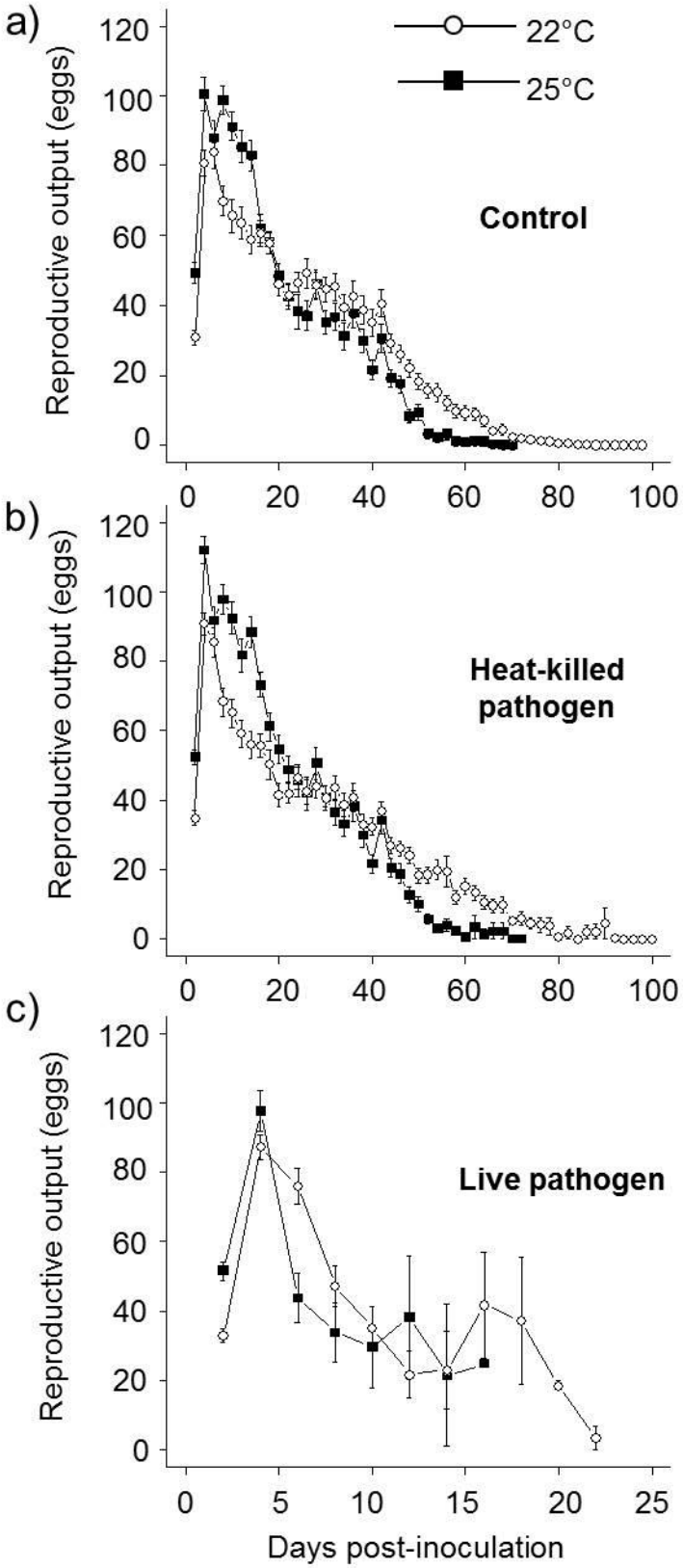

Figure 3.8 Age-specific fecundity patterns of *Drosophila* under different temperature and pathogen treatments. All error bars are standard errors of the mean.

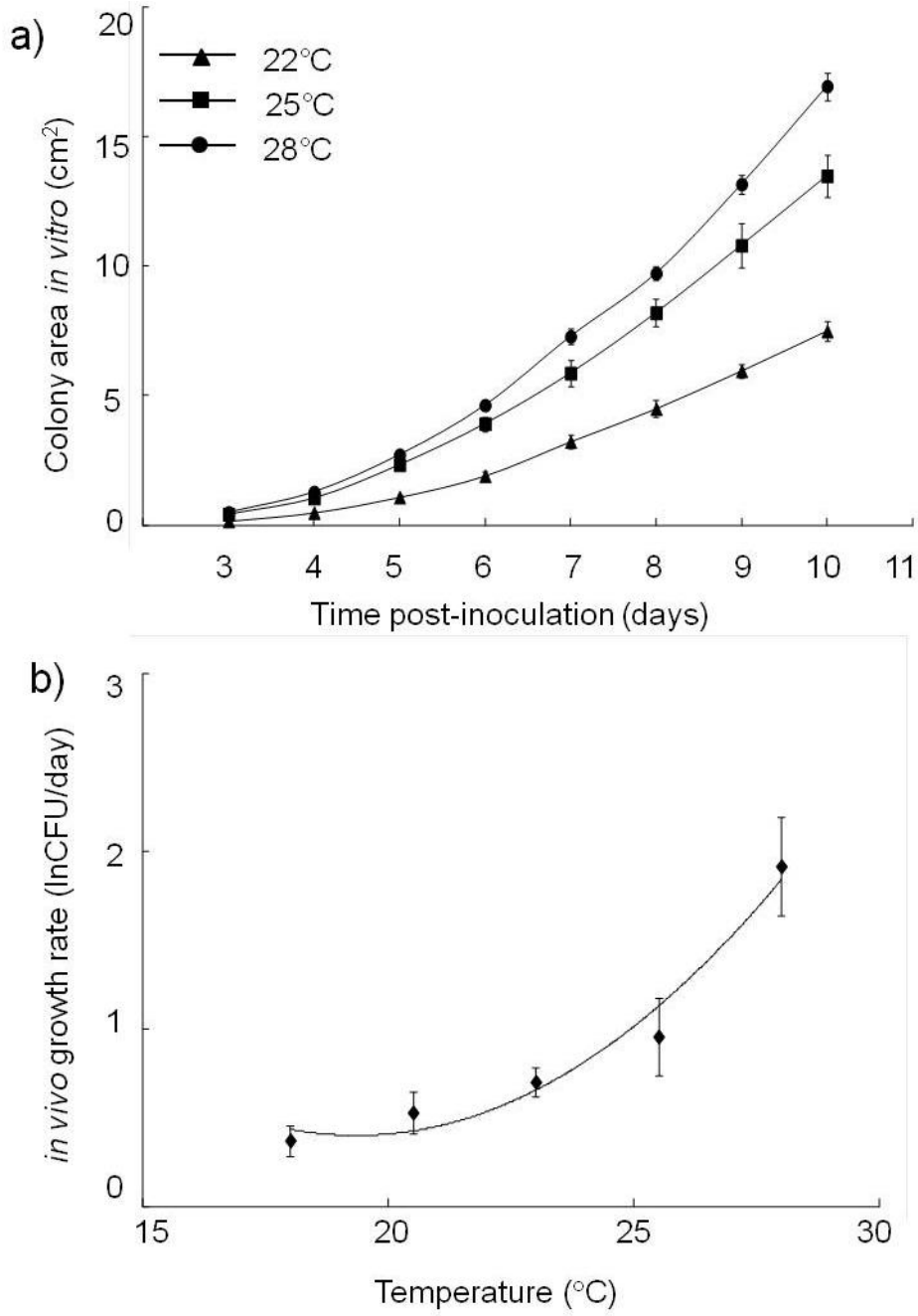

Figure 3.9 Moving to colder temperatures is detrimental to the fungal parasite. a) *in vitro* colony growth of *Metarhizium robertsii* at 22, 25 and 28°C (Post-hoc Tukey's HSD tests revealed all pairwise comparisons were significant at p = 0.0001). b) *in vivo* rate of growth of fungal pathogen at 18, 20.5, 23, 25.5 and 28°C. Samples of live flies were taken at 3-4 day intervals and parasite load was established by counting the number of colony forming units (CFUs) on replicate fungal media plates. The fitted line is least squares polynomial regression of natural-log transformed CFU counts between the first sampling point to peak CFU. Error bars are standard errors of the mean.

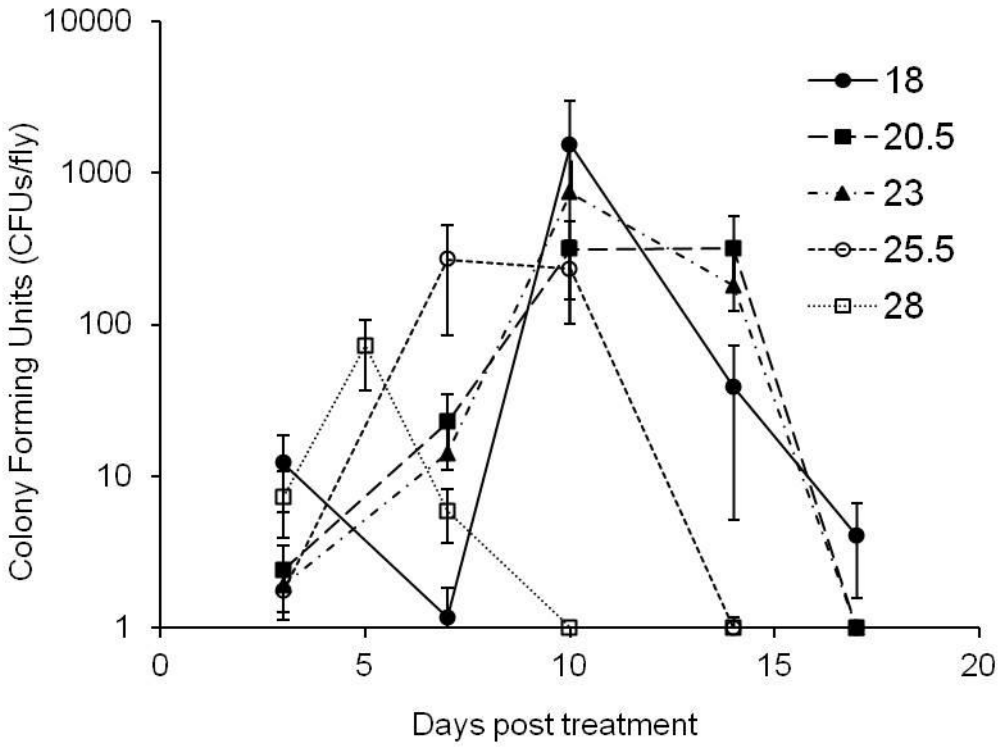

Figure 3.10 Effect of temperature on parasite load up to 17 days post-inoculation. Parasite load was estimated by counting the number of colony forming units (CFUs) recovered from surviving infected flies at different time points. Error bars are standard errors of the mean.

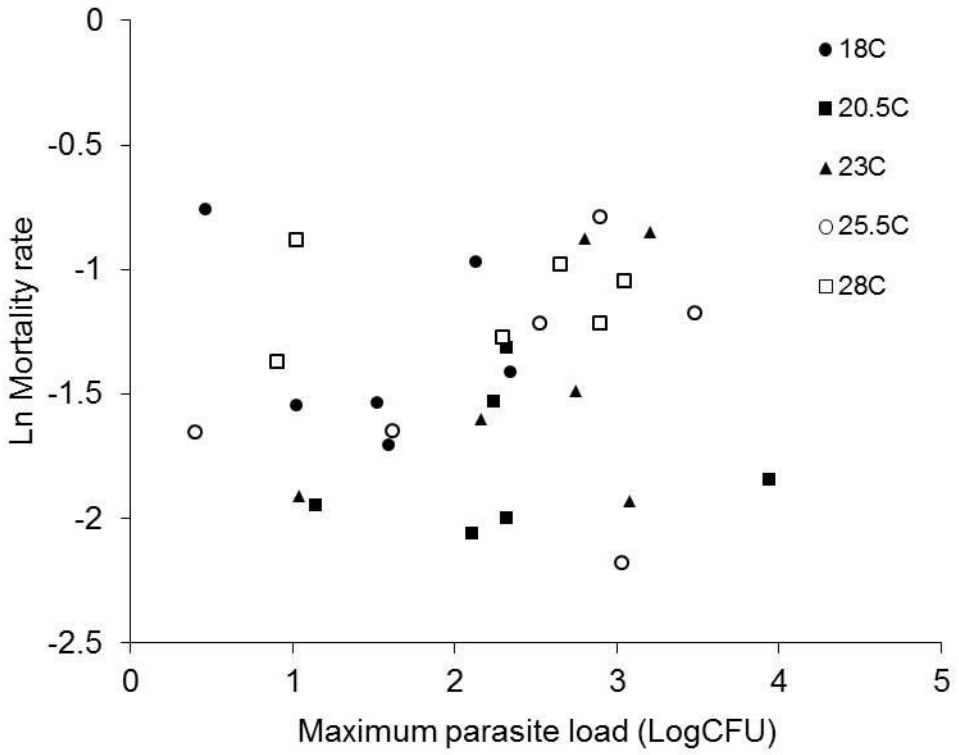

Figure 3.11 Temperature did not influence the relationship between parasite load and host mortality (tolerance) of flies infected with *Metarhizium*. Plotted points are maximum CFU counts observed from each replicate cage population at each temperature regime. Mortality rates were estimated from the number of deaths from the same fly populations that provided parasite load data.

Table 3.1 Analysis of variance terms and significance for the effect of pathogen and temperature on two fitness measures. Intrinsic rate of increase was estimated using each treatment combination within each block using the number of eclosed pupae produced at each collection interval (n=30). Lifetime reproductive success was estimated as the total number of eclosed pupae over the entire lifetime for each female. Note that we excluded all females that produced fewer than 5 eclosed pupae prior to the analysis (n=233).

Response variable	**Effect**	**Test statistic and p value**
Intrinsic rate of increase (*r*)	Pathogen	$F_{2,24} = 2.34$, $p = 0.118$
	Temperature	$F_{1,24} = 19.4$, $p = 0.0002$
	Pathogen × Temperature	$F_{2,24} = 1.00$, $p = 0.384$
Lifetime reproductive Success (LRS)	Pathogen	$F_{2,227} = 1.08$, $p = 0.341$
	Temperature	$F_{1,227} = 3.97$, $p = 0.047$
	Pathogen × Temperature	$F_{2,227} = 1.87$, $p = 0.157$

Table 3.2 Mixed effects model terms and significance for overall age-specific fecundity models. Individual females were fitted as random intercepts. We excluded the first egg collection interval post inoculation (day 0-2) in the analysis because accurate model fitting was inhibited by the low levels of fecundity seen in these newly emerged flies.

Fixed effect	**Test statistic and p value**
Age	$F_{1,4923} = 218.3$, $p < 0.0001$
Pathogen	$F_{2,248} = 24.0$, $p < 0.0001$
Temperature	$F_{1,248} = 27.7$, $p < 0.0001$
Age × Pathogen	$F_{2,4923} = 144.6$, $p < 0.0001$
Age × Temperature	$F_{1,4923} = 77.3$, $p < 0.0001$
Pathogen × Temperature	$F_{2,248} = 0.43$, $p = 0.65$
A × P × T	$F_{2,4923} = 15.5$, $p < 0.0001$

Table 3.3 Mixed effects model terms and significance for individual age-specific fecundity models. Individual pathogen treatments were fitted as separate mixed effects models. Mixed effects model was fitted to the entire data set after excluding the first egg collection interval post inoculation (day 0-2) as in Table 3.2. Individual females are fitted as random intercepts.

Fixed effect	**Test statistic and p value**		
	Control	Heat-killed fungus	Live fungus
Age	$F_{1,2242} = 171$, $p < 0.0001$	$F_{1,2444} = 264$, $p < 0.0001$	$F_{1,237} = 107$, $p < 0.0001$
Temperature	$F_{1,83} = 17.8$, $p < 0.0001$	$F_{1,84} = 33.0$, $p < 0.0001$	$F_{1,81} = 3.4$, $p = 0.069$
Age × Temperature	$F_{1,2242} = 52.9$ $p < 0.0001$	$F_{1,2444} = 92.7$ $p < 0.0001$	$F_{1,237} = 9.8$, $p = 0.002$

Table 3.4 Mixed effects model terms and significance for age-specific parasite load. Model was fitted to log-transformed CFU data set after excluding measurements made on day 5 which only contained estimates for 28°C treatment. Individual cages were fitted as random intercepts.

Fixed effect	**Test statistic and p value**
Age	$F_{4,103} = 3.89$, $p = 0.007$
Temperature	$F_{1,28} = 0.17$, $p = 0.68$
Age × Temperature	$F_{4,103} = 3.40$, $p = 0.012$

Table 3.5 Mixed effects model terms and significance for the effect of temperature on the relationship between parasite load and host mortality (tolerance). Model was fitted to natural-log transformed mortality rates. We used log-transformed CFU data set after excluding measurements made on day 5 which only contained estimates for 28°C treatment. Parasite load was log10 transformed. Age and temperature were fitted as categorical variables. Individual cages were fitted as random intercepts.

Fixed effect	**Test statistic and p value**
Parasite load	$F_{1,98} = 4.57$, $p = 0.035$
Temperature	$F_{4,25} = 3.31$, $p = 0.026$
Age	$F_{5,98} = 107$, $p < 0.0001$
Parasite load x Temperature	$F_{4,98} = 0.96$, $p = 0.43$

Chapter 4 Fitness-associated maternal effects on meiotic recombination and life history in response to fungal infections

Weihao Zhong and Nicholas K Priest

Abstract

Hosts mount a multitude of responses to mitigate the fitness loss due to infections. However, we do not know the relationship between these responses and the fitness of the host, or whether such effects extend beyond a single host generation. Theory predicts that fitness-associated maternal effects could accelerate the rate of adaptive evolution, particularly when low fitness mothers produce offspring with higher rates of meiotic recombination and when maternal fitness positively influences offspring life history. Here we provide evidence that both can occur when the fruit fly, *Drosophila melanogaster*, is infected with a fungal parasite, *Metarhizium robertsii*. We show that meiotic recombination rate of the offspring is negatively correlated with maternal reproductive success when both generations experienced live pathogen exposure. We also show that exposure to live fungal pathogen generates a positive relationship between maternal and offspring reproductive success. These findings indicate that hosts can generate fitness-associated maternal effects against infections which have the potential to accelerate adaptive evolution.

Author contributions:
WZ: conceived, designed and conducted the experiment, analysed the data and prepared the manuscript. NKP: conceived and designed the experiment and improved the manuscript

4.1 Introduction

It is well known that infections often reduce host fitness (Lehmann 1993). But the responses hosts mount against invading parasites might also depend on their fitness. Intuitively, high fitness hosts should respond differently than low fitness hosts because their relative competitive advantage is accentuated by infection. However, the occurrences of such fitness-associated responses and their potential impacts on the evolutionary process remain poorly understood.

Host and parasite interactions are likely to impact on the mechanism that help to generate genetic diversity over multiple generations. Previous studies have shown that meiotic recombination is sensitive to the physiological state of the organism, which are elevated during periods of genetic or environmental stress (Parsons 1988; Hoffmann & Hercus 2000; Badyaev 2005; Priest et al. 2007; Zhong & Priest 2010). Because of the detrimental effect of infection on host fitness, we would expect strong host response in meiotic recombination. Indeed, parasite exposure is known to greatly enhance the rate of homologous recombination in plants (Lucht et al. 2002; Kovalchuk et al. 2003). Parental parasite exposure history can also influence meiotic recombination in their offspring. Significant reduction in maternal fitness due to infection could indicate a susceptible genotype. Thus, in chronically infected lineages it could be advantageous for infected parents to stimulate recombination in their offspring, since this will tend to break down the existing maladapted allele combinations and generate fitter or more resistant combinations. For example, in plants, homologous recombination has been shown to be remain elevated for several generations after parasite exposure in a single generation (Kathiria *et al.* 2010; but also see Molinier *et al.* 2006). In animal studies, meiotic recombination appears to increase with parasite load (Camacho *et al.* 2002) and experimental co-evolution with parasites (Fischer & Schmid-Hempel 2005; Kerstes et al. 2012); however, whether maternal response to infection also induce fitness-associated effects on recombination in the offspring has not been directly tested.

Theory shows that fitness-associated and trans-generational influences on recombination readily evolve and increase the rate of adaptive evolution (Hadany & Beker 2003a; Hadany & Beker 2003b; Agrawal et al. 2005; Hadany & Otto 2009). A general feature of these models is that unlike classic models of recombination in which an uniform rate of recombination is assumed for the entire population,

recombination in fitness-associated recombination (FAR) models is enhanced in low fitness individuals but reduced in high fitness individuals (Hadany & Beker 2003a). In particular, models of diploid organisms also predict that the rate of recombination in the offspring should be negatively associated with maternal fitness (Agrawal et al. 2005).

FAR is not the only mechanism by which parasites can influence the rate of adaptive evolution of the host. Because parasites directly compete for host resource and cause the host to mount costly immune responses (Schmid-Hempel 2003), infections alter the pattern of host resource allocation to reproduction (Minchella 1985; Agnew et al. 2000; Hurd 2001). In turn, maternal reproductive decisions influence both the number and size of propagule, which are important for many early-age life history traits of offspring such as viability and developmental rate (Bernardo 1996; Mousseau & Fox 1998; Brown & Shine 2009). Such fitness-associated response to infection on life history could have significant impact on the rate of adaptive evolution. Specifically, quantitative genetics models of maternal effects predict that positive effects of maternal phenotype on offspring phenotype (i.e. positive maternal effect coefficient) will accelerate the evolutionary response to directional selection (Kirkpatrick & Lande 1989; Wolf et al. 1998). This would be the case if high fitness mothers also produce high fitness offspring. Conversely, negative maternal effect coefficients are predicted to impede the evolutionary response (Kirkpatrick & Lande 1989; Wolf et al. 1998), which would be the case if low fitness mothers produce high fitness offspring, and vice versa.

Here we conducted simultaneous experiments to determine a) how infection alters fitness-associated meiotic recombination and life history, b) the extent to which maternal effects contribute to these patterns, and c) whether the parasite needs to be live to stimulate fitness-associated responses. The fruit fly, *Drosophila melanogaster,* as the host for the generalist insect fungal pathogen, *Metarhizium robertsii,* is an ideal system with which to test these questions. Topical application of *Metarhizium* spores are known to incur fitness costs in *Drosophila* (Zhong et al. 2013). The fruit fly is well-suited for large-scale empirical studies of life history and fitness (Prasad & Joshi 2003; Nicholas K Priest et al. 2008), and it has a long history in the study of stress induced-recombination (Plough 1917; Bridges 1929; Neel 1941; Priest et al. 2007; Zhong & Priest 2010; Tedman-Aucoin & Agrawal 2012).

4.2 Materials and Methods

4.2.1 Fly stocks and backcrossing

Strains of *D. melanogaster* with dominant phenotypic markers for the second chromosome Kruppel ($Kr^{If\text{-}1}$) and Black cell (Bc^{1}) were obtained from Bloomington Drosophila Stock Centre ($b^{1}Kr^{If\text{-}1}$, stock number 4194; $Bc^{1}fj^{1}wt^{1}$, stock number 1036). The Kr-Bc marker interval was chosen as a previous study has reported strong recombination responses when flies were subjected to mating-related stress (Priest et al. 2007). We first backcrossed the marker strains with the wild-type Dahomey strain (provided by Dr Stuart Wigby, University of Oxford), which originated from West Africa and had been maintained in large population cages with overlapping generations for more than 30 years (Partridge & Andrews 1985). Virgin Dahomey females were initially crossed with homozygous marker males (Bc+/Bc+ or Kr+/Kr+). Virgin females heterozygous for the two markers (Bc+/++ or Kr+/++) were collected from the F1 offspring and crossed with wild-type males. This was repeated for 5 and 7 generations resulting in heterozygous marker lines that were 99.2% and 96.9% Dahomey background for Black cell and Kruppel, respectively. All stocks and experimental animals were maintained at 25 °C and 40 % relative humidity in standard *Drosophila* vials (28.5 × 95 mm) on oatmeal-molasses-agar media containing a single grain of live baker's yeast. An antifungal agent (Nipagin) was added to the cooked media to inhibit the growth of naturally-occurring saprophytic fungi. To ensure uniform larval environment, we maintained the backcrossed marker lines at a low egg density of 50 eggs/vial for the last four generations prior to the start of the experiment.

4.2.2 Fungal culture and infection

Isolate 2575 of *M. robertsii* (Ascomycota: Hypocreales; previously classified as *M. anisopliae* strain ME1; Bischoff *et al.* 2009) was obtained from the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, United States Department of Agriculture. We cultured the fungus on quarter-strength Sabouraud Dextrose Agar media with additional yeast extract (SDAy). Fresh SDAy plates were inoculated by spreading fungal spore suspension (4×10^{7}spores/ml) and incubated at 28°C for two weeks. Mature sporulating cultures were transferred to 4°C for storage until required. We assessed the viability of spores by examining the *in vitro*

germination success of spores under a low power microscope with only cultures achieving at least 95% germination success used for experiments. Prior to inoculation, spores were collected from multiple culture plates using sterile plate spreaders. Pathogen treatments were performed on groups of 20 flies in 250ml conical flasks. For live pathogen (LP), we transferred the flies without CO_2 anaesthesia to the flask containing 900μg of live spores and gently shook for 10 seconds. For heat-killed pathogen (HP), the files were treated identically in a flask containing an equal dose of inactivated spores (autoclaved at 121 °C for 15 min). No pathogen (NP) flies were treated by gentle shaking in an empty flask for 10 seconds.

4.2.3 Experimental procedures

We manipulated pathogen exposure over two generations (F_0 Mother and F_1 Daughter) and assessed the life history and meiotic recombination responses in the F_1 daughter generation by examining the grandoffspring (F_2) (**Figure 4.1**). Briefly, we first collected adult virgin females heterozygous for Black cell (Bc+/++) over 24 hours from the backcrossed marker line and held them in fresh vials at a density of 10 flies/vial for 48 hours. Without CO_2 anaesthesia, we introduced 10-15 males heterozygous for Kruppel (Kr+/++) into each vial at female adult age day 3 and removed them after 24 hours. Mated females were kept in vials for a further 24 hours before pathogen treatment. At adult age day 5, females were treated according to one of three pathogen treatments as described above and transferred to individual vials in randomly assigned positions across six blocks. The food vials were replaced every two days thereafter until day 6 post-inoculation. A total of 240 mothers were generated (NP=81, HP=79, LP=80).

We collected doubly heterozygous virgin daughters (Bc+/Kr+) from the maternal progeny produced during the 2-4 day post-inoculation period (within a 36 hour eclosion window), as meiotic recombination is absent in male *D. melanogaster* and because previous experiments had shown that infected flies first begin to display visible signs of *Metarhizium* infection from two days after the initial exposure (**Chapter 3**; Hunt et al., submitted). Daughters were kept with their maternal sisters (both full and half-sibs) in fresh vials for two days. At adult age day 3, daughters were transferred to individual vials containing a single tester wild-type Dahomey male (++/++) for 24 hours. At adult age day 5, we individually treated daughters with one of three pathogen treatments in glass universal tubes and randomly assigned

them to positions across 12 blocks, with all daughters from the same mother assigned to the same treatment (i.e. maternal families nested within treatment). Similar to the maternal generation, all vials were replaced every two days until day 6 post-inoculation. Overall, in combination with maternal treatments, a total of seven pathogen treatment combinations were generated that included a total of 505 daughters (**Figure 4.1**).

4.2.4 Fitness and recombination measurements

The number of eggs produced by each mother and daughter during every 2-day collection interval was counted under a dissection microscope until day 6 post-inoculation. Used vials from each egg collection were kept for up to 20 days and the numbers of eclosed pupae were counted. Because female fruit flies achieved between 80 and 95% of their lifetime pupae production in the first six days post-treatment under our experimental conditions (**Chapter 3**), our measure of total fecundity over this period is comparable to the lifetime reproductive success (LRS) for both mothers and daughters. We also estimated the intrinsic rate of increase or the Malthusian parameter (*r*) for the daughter generation, which is a better measure of fitness when the population is growing or declining (Charlesworth 1994; Brommer et al. 2002). Using the number of hatched pupae produced at each collection interval, we estimated *r* for each mother × daughter treatment combination within each block by solving numerically the discrete form of the Euler-Lotka equation: $1 = \Sigma e^{-rx} l_x m_x$, where *x* is the age class, l_x is the probability of surviving from age class *x* to age *x* +1, and m_x is the expected number of offspring for a female in age class *x* (n=84; Charlesworth 1994).

To estimate the rate of meiotic recombination between Black cell and Kruppel markers, all progeny of the daughters were first stored at -20°C, and over a period of six months, scored for the presence and absence of Black cell and Kruppel phenotypes under a low power dissection microscope. We scored the proportion of recombinant phenotypes (BcKr/++ and ++/++) and non-recombinant parental phenotypes (Bc+/++ and Kr+/++). A total of 82523 grandoffspring were scored. For representation in figures, we first converted the proportions of recombinants to linkage map units using Haldane's mapping function (Haldane 1919). We then calculated the recombination density (cM/Mb), a measure that normalizes recombination frequency by the physical distance along the chromosome (Wilfert et

al. 2007), for each daughter by dividing map units in cM by the estimated physical distance between the markers in mega base pairs (Kr–Bc: 7.3 Mb, FlyBase).

4.2.5 Statistical analyses

All statistical analyses were performed using R version 2.15 (R Development Core Team 2010). In total, 472 daughters were included in the analysis; we censored 33 daughters from all analyses because they did not lay any viable eggs or were accidentally lost during the experiment. Because we did not create fully factorial combinations of live and heat-killed pathogen treatments, the two data sets were analysed separately. We used Generalized Linear Mixed Models (GLMMs; *glmer ()* function in R package "lme4") to assess the effect of maternal and daughter pathogen exposure on daughter age-specific meiotic recombination (the proportion of recombinant grandoffspring scored). GLMMs are particularly appropriate for our data as it displays both temporal autocorrelation and non-normal distributions (Bolker *et al.* 2009). The full model included maternal and daughter pathogen treatment, age, maternal total fecundity with all their interaction terms and block as fixed effects, with individual daughters nested within their mothers as random effects. Separate models were fitted to individual treatment to assess the significance of the slope of the relationship between maternal total fecundity and daughter recombination. In all cases, statistical significance was established by comparing nested models with different fixed effect model structures using log likelihood ratio tests (following Crawley 2007).

We used Linear Mixed-effects Models (LMEs; *lme ()* function in R package "nlme") to assess the effect of maternal and daughter pathogen exposure on daughter life history. For daughter total fecundity, we fitted models that included maternal and daughter pathogen treatments with all their interaction terms and block as fixed effects, with individual mothers as a random effect, which accounts for similarity among maternal sisters. For daughter age-specific fecundity (pupae and eggs), we fitted models that included maternal and daughter pathogen treatments, age, all of their interaction terms and block as fixed effects, with individual daughters nested within mothers as random effects. For the relationship between maternal total fecundity and daughter fitness measures (total fecundity and age-specific fecundity), we added maternal total fecundity and all their interaction terms to their respective simpler models as outlined above. Planned *a priori* contrasts within the full model

were used to assess the difference in the slope of the relationship between mother and daughter total fecundity among treatments. For intrinsic rate of increase, the full model included maternal treatment, daughter treatment, and their interaction as fixed effects, with block as a random effect.

4.3 Results

4.3.1 Fitness associated maternal effect on recombination

We found that pathogen exposure significantly altered the relationship between maternal fitness and daughter recombination. In fruit fly populations that were infected over two generations, low fitness mothers produced daughters with higher recombination rate than high fitness mothers (Day 2-4: Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, χ^2_1=6.24, p=0.012; **Figure 4.2 and 4.3**). Unlike maternal total fecundity, daughter age-specific pupae production was not associated with recombination at any age interval (Daughter Fecundity × Maternal Treatment × Daughter Treatment × Age, χ^2_2 = 0.14, p = 0.93).

Further analysis revealed that the strength and direction of the fitness-association depended on the maternal and daughter pathogen treatments as well as the age post-inoculation at which recombination was measured (Maternal Total Fecundity × Maternal Treatment × Daughter Treatment × Age, χ^2_2=13.96, p=0.00093; **Figure 4.2 and 4.3**). Specifically, at 2-4 days post-inoculation, a negative relationship between daughter recombination frequency and maternal total fecundity was only apparent when both the mother and daughter were treated with the live pathogen (MLP+DLP, z = 2.1, p = 0.036), but not when neither were exposed to the live pathogen (MNP+DNP, z =1.6, p = 0.11; **Table 4.1**). There was no evidence for significant negative relationships between maternal fitness and daughter recombination in the remaining age intervals (Day 0-2: Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, χ^2_1=3.60, p=0.058; Day 4-6: Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, χ^2_1=0.40, p=0.53).

In contrast, by using simpler models that excluded maternal total fecundity, we found no evidence that maternal or daughter pathogen treatment affected mean recombination frequency at any age (Maternal Treatment × Daughter Treatment × Age, χ^2_2=2.30, p=0.32; **Figure 4.4a**), though the proportion of recombinant offspring was marginally increased at late age intervals (Age, χ^2_2=5.66, p=0.059). Lastly, we

found no evidence that exposure to heat-killed pathogen affected either mean daughter recombination (Maternal Treatment × Daughter Treatment × Age, χ^2_2=1.44, p=0.49; **Figure 4.4b**) or the relationship between maternal fitness and daughter recombination (Maternal Total Fecundity × Maternal Treatment × Daughter Treatment × Age, $\chi^2_2 = 3.76$, p = 0.15).

4.3.2 Fitness associated maternal effect on life history

We found that pathogen exposure significantly altered the relationship between maternal and daughter fitness. In fruit fly populations that were infected over two generations, there was a trend for low fitness mothers to produce daughters with low fitness (Maternal Treatment × Daughter Treatment × Maternal Total Fecundity, $F_{1,93}$ =5.99, p = 0.016; **Table 4.2**; **Figure 4.5 and 4.6**). Moreover, the relationship between maternal and daughter fitness could not be explained by the larval environment of the daughter as the three-way interaction term remained significant even after accounting for larval density in the model (Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, $F_{1,92}$ =8.81, p = 0.0038).

Further analysis revealed that while under the control no pathogen conditions high fitness mothers produced lower fitness daughters (MNP+DNP, t = 2.15, p = 0.034; **Figure 4.5 and 4.6**), in all live pathogen treatments, the fitness of mothers and daughters tended to be positively correlated, though the relationship was only significantly positive in live pathogen treated mother and control daughter treatment (MLP+DNP, t = 2.06, p = 0.042; **Table 4.3**). Furthermore, this pattern was primarily driven by the effect of maternal fitness on daughter egg and pupae production during the first two days post-inoculation (Eggs: Day 0-2 Maternal Total Fecundity × Maternal Treatment × Daughter Treatment: $F_{1,93}$ = 9.85, p = 0.0023; Pupae: Day 0-2 Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, $F_{1,93}$ =8.86, p = 0.0037; Day 2-4, and day 4-6 are not significant; stats not shown).

In contrast, although we confirmed that direct exposure to live pathogen in daughters has negative consequences for their fitness (Daughter Treatment, $F_{1,97}$ = 6.58, p = 0.012), we found no evidence for any effect of maternal exposure, either as main treatment or interaction effect on mean daughter fitness (Maternal Treatment, $F_{1,97}$ =1.31, p = 0.25; Maternal Treatment × Daughter Treatment, $F_{1,97}$ = 0.15, p = 0.70). This pattern was also confirmed by age-specific analysis of daughter pupae production; direct exposure to live pathogen caused fecundity reduction 2-4 days

after inoculation, which became more prominent during the 4-6 day interval (Daughter Treatment × Age, $F_{2,550} = 14.04$, $p < 0.0001$; **Figure 4.7a**). Interestingly, both maternal and direct exposure to live pathogen tended to enhance the intrinsic rate of increase of daughters, resulting in MLP+DLP females achieving the highest *r* (Maternal Treatment, $F_{1,33} = 3.64$, $p = 0.065$; Daughter Treatment, $F_{1,33} = 6.80$, $p = 0.014$; **Figure 4.8**). Lastly, we found no evidence that exposure to heat-killed pathogen affected either mean daughter fitness (Maternal Treatment × Daughter Treatment, $F_{1,92} = 0.35$, $p = 0.56$; **Figure 4.7b**), or the relationship between maternal and daughter fitness (Maternal Total Fecundity × Maternal Treatment × Daughter Treatment, $F_{1,89} = 0.78$, $p = 0.38$).

4.4 Discussion

Exposure to parasites could induce fitness-associated effects on host meiotic recombination and life history that extend beyond a single generation. Such effects could accelerate the rate of adaptive evolution when low fitness mothers produce offspring with higher rates of meiotic recombination (Agrawal et al. 2005), and when maternal fitness positively influence offspring life history traits (Kirkpatrick & Lande 1989). We found evidence that in *Drosophila* both mechanisms could operate, though the effects are likely to be highly context dependent. These results provide empirical support for the theory of fitness-associated recombination (Hadany & Beker 2003b; Agrawal et al. 2005) and show that incorporating maternal fitness in maternal effect studies can yield novel insights.

4.4.1 Fitness-associated maternal effect on meiotic recombination

Models of fitness-associated recombination incorporating maternal effects predict that maternal fitness should be negatively correlated with meiotic recombination in the offspring (Agrawal et al. 2005). Though many studies of stress-induced recombination are generally consistent with this model prediction (reviewed in Parsons 1988; Hoffmann & Hercus 2000; Badyaev 2005), few studies have examined the possibility that maternal stress exposure can induce recombination in the offspring (though there are examples in plants (Molinier et al. 2006; Kathiria et al. 2010)), or investigated the relationship between reproductive success and recombination within experimental stress treatments (Zhong & Priest 2010;

Appendix I). We found that live pathogen exposure altered the relationship between maternal fitness and daughter recombination. In particular, when both maternal and daughter generations were exposed to the live pathogen (MLP+DLP), low fitness mothers produced daughters with elevated recombination rate during the day 2-4 interval.

Our results suggest that in a parasite-rich environment the adaptive potential of a population might be enhanced through fitness-associated recombination (Hadany & Beker 2003a; Agrawal et al. 2005; Hadany & Otto 2009). This finding is particularly intuitive from a lineage perspective. Succumbing to infection and suffering significant reduction in reproductive success by the same strain of fungal parasite across two generations is likely to be a reliable indicator of a low quality or susceptible genotype. Conversely, maintaining high levels of reproduction even while being infected might serve as an 'honest signal' of genetic quality. Increased meiotic recombination in the daughters of such lineages will tend to break up the existing allele combinations and result in an increase in fitness (Hadany & Beker 2003b; Agrawal et al. 2005). Of course, our results are also compatible with straightforward mechanistic explanations. For example, if low fitness mothers are affected by infection to such an extent that they are unable to buffer physiological stress and suffer much more DNA damage, meiotic recombination could increase as an attempt to repair damage and restore genome fidelity (Szostak et al. 1983; Cromie & Smith 2007).

It is also apparent from our results that fitness-associated maternal effects on recombination are highly age and environment-dependent. The fact that we did not detect a significant negative relationship until the second age interval (day 2-4 post inoculation) is consistent with the known infection process of *Metarhizium* in *Drosophila* e.g. reductions in host survival and reproduction are detectable from 48 hour post-inoculation (Zhong *et al.* 2013; **Chapter 3**). The age-specific nature of the maternal effect could indicate that the daughters were stimulated to respond only once the infection had become established. This interpretation is supported by our finding that only live pathogen treatment induced fitness-associated recombination, and the previous findings that such relationships are only detectable in 'high-stress' treatments (Zhong & Priest 2010). It has previously been suggested that increased sex and recombination should be expected when parasites are vertically transmitted (Rice 1983). Although there is no evidence of vertical transmission in *Metarhizium*

(see Caveats), the fact that we only found evidence of a negative relationship when the exposure was maintained for two generations suggest that the similarity between the maternal and offspring parasite environments is an important factor affecting meiotic recombination, rather than the transmission method *per se*. Indeed, repeated stress exposure over multiple generations appear to be a general requirement for maintaining strong transgenerational effects in plants (Boyko & Kovalchuk 2010). More generally, our results show that the interplay between past and current stress is important in shaping the expression of maternal effects (Plaistow et al. 2006; Jansen et al. 2011).

However, it is important to note that we also found within the same 2-4 day interval evidence for a fitness-associated maternal effect on recombination in the opposite direction. When only daughters were exposed to the live pathogen (MNP+DLP), high fitness mothers produced daughters that had higher recombination rates than those from low fitness mothers, which is a pattern predicted to be much rarer and have different evolutionary consequences (Agrawal et al. 2005). Thus, it is unclear whether parasite-induced fitness-associated maternal effect on recombination will always facilitate adaptive evolution.

4.4.2 Fitness-associated maternal effect on life history

Although many studies have examined genetic and phenotypic correlations for many maternal and offspring traits (reviewed in Räsänen & Kruuk 2007), few have assessed the direct correlation between maternal and offspring reproductive success or examined the effect of pathogen exposure on the correlations. We found that maternal exposure to the live pathogen generated a positive relationship between maternal and daughter reproductive success (MLP+DNP). By contrast, under the control conditions (MNP+DNP) daughters whose mother achieved high reproductive success had lower reproductive success themselves i.e. a strong negative correlation.

Our results suggest that in a parasite-rich environment maternal fitness has a positive influence on daughter life history, which is likely to enhance the response to directional selection in our experimental population (Kirkpatrick & Lande 1989). Positive maternal effect coefficients have previously been documented in wild populations (reviewed in Räsänen & Kruuk 2007), and they are generally expected to be beneficial for tracking the optimal phenotype following environmental shifts (Hoyle & Ezard 2012). This positive relationship is likely to be the result of

infections altering host resource allocation (Minchella 1985; Agnew et al. 2000; Hurd 2001). Through direct competition and triggering costly immune defences, fungal infections increased the demand on host resources and caused reductions of reproductive output in all females, particularly from day 2-4 post-inoculation onwards (**Figure 4.7a**). However, the trade-offs between immune functions and life history traits are expected to be strongest in low fitness hosts (e.g. as a result of food-restriction, McKean et al. 2008). Thus, low fitness mothers are likely to be disproportionately affected by infection, and as a result, produce poorly provisioned daughters which have lower reproductive success themselves as adults.

Although we did not measure it in this study, parasite-induced maternal effects influencing the reproduction of daughters are likely to be mediated by egg size (Bernardo 1996). In *Drosophila*, egg size influences egg viability, developmental rate, hatchling weight and feeding rate (Azevedo et al. 1997). Previous studies of parental larval diet effect have found that female fruit flies reared on nutritionally poor diets produced relatively large eggs (Vijendravarma et al. 2010; Valtonen et al. 2012). Exposure to parasitic mites have been also shown to produce fewer but larger eggs in Damselflies (Rolff 1999). If infected mothers alter the provisioning to her eggs, then it could also help explain another unexpected result. Although it was not significant statistically, we found a trend for greater intrinsic rate of increase in daughters whose mothers were exposed to the live pathogen, particularly when the exposure was maintained in both generations (MLP+DLP; **Figure 4.8**). Faster rate of pre-adult development and greater early-age reproductive output as the result of increased egg size would naturally elevate the intrinsic rate of increase (Charlesworth 1994; Brommer et al. 2002). Such maternal 'priming' for increased reproduction in offspring early in life is consistent with the general expectation that parasitized host should increase its current reproductive effort (Minchella 1985; Forbes 1993; Agnew et al. 2000), which might be a beneficial strategy to ensure some reproductive success in a pathogen-rich environment. However, a previous study in *Drosophila* found no effect of bacterial challenge on egg size (Linder & Promislow 2009). Therefore, further experiments measuring egg size are required to examine this possibility more closely.

In contrast, our results suggest that under the control conditions employed in this experiment the evolutionary response to directional selection is likely to be impeded in our fly population, at least in the first few generations (Kirkpatrick &

Lande 1989). Negative influences of maternal reproduction, in particular clutch size, on offspring life history traits are well documented (Falconer 1965; Janssen et al. 1988; Schluter & Gustafsson 1993; McAdam & Boutin 2004). It has been suggested that negative maternal effect coefficients are beneficial in populations under stable environments as it tend to minimise deviations from the optimal phenotype thus maximises fitness (Hoyle & Ezard 2012). This negative maternal effect coefficient can be explained by the classic trade-off between number and quality of offspring (Smith & Fretwell 1974; Stearns 1992; Bernardo 1996); where high fitness mothers sacrifice the individual quality of their offspring by producing greater numbers of them (Einum & Fleming 2000), whereas low fitness mothers produce fewer but higher quality offspring, which increase their daughters' fecundity as adults.

4.4.3 Caveats

It is surprising that we did not find any significant effect of maternal parasite exposure on mean meiotic recombination. Previous studies of *Drosophila* have often reported stress-induced recombination by a variety of factors including temperature, age, nutrition, mating, and mutation (Plough 1917; Bridges 1929; Neel 1941; Priest et al. 2007; Zhong & Priest 2010; Tedman-Aucoin & Agrawal 2012). It is unlikely that we lacked sufficient statistical power as the sample sizes for both the number of replicate females (72 daughters/treatment) and the number of offspring scored (163 flies scored/daughter over 6 days) were comparable to Priest et al. (2007), which used the same Kr-Bc markers. It is important to note however, that because we only monitored one marker interval, other genomic regions might show greater responses; intriguingly, many *Drosophila* immune gene clusters are located in areas of genome with high rates of background recombination (Wegner 2008). Another potential problem is that we could have underestimated the proportion of recombinant offspring in infected treatments because the Black cell mutation compromises the antifungal immunity of the fly by interfering with melanisation reaction (Grell et al. 1980; De Gregorio et al. 2002), which could result in fewer recombinant offspring (Kr Bc /+ +) surviving long enough to be scored. However, this is unlikely to be the case for several reasons. First, there is no evidence that *Metarhizium* can be vertically transmitted from female to her offspring in *Drosophila*. Second, melanisation reaction does not appear to be critical for antifungal immunity in fruit flies (Leclerc et al. 2006; Tang et al. 2006). Third, independent survival experiment

did not find Black cell heterozygous Dahomey flies (Bc + /+ +) to be more susceptible to *Metarhizium* infections (**Figure 4.9**).

Finally, the magnitudes of maternal effects reported in this study are likely to be conservative. While we conducted all experiments at 25°C, the preferred temperature for healthy laboratory-adapted *Drosophila* (**Chapter 3**; Sayeed & Benzer 1996), fruit flies infected by *M. robertsii* would seek out cooler temperatures if allowed the opportunities to thermoregulate, entailing significant impacts on their life history (**Chapter 3**; Huey et al. 1995; Gilchrist & Huey 2001) and perhaps even meiotic recombination (Plough 1917; Zhong & Priest 2010). Moreover, maternal effects are generally strongest early in the life of the offspring (Rossiter 1996; Boulinier & Staszewski 2008). Although we allowed mothers 2-4 days post-inoculation in which to alter provisioning or transfer cues to the developing eggs (cf. 24 hours post-infection in Linder & Promislow 2009), the daughters were not treated until they were 4-5 day old adults. We would expect even greater maternal effects if daughters were exposed to the pathogen earlier in life e.g. as larvae.

4.4.4 Conclusion

Our study shows that parasite exposure can have complex fitness-associated effects over multiple generations. Both an individual's capacity to generate genetically diverse offspring and its reproductive success are partially determined by its mother. Crucially, the individual response to parasites depend on maternal fitness, which can be decoupled from the 'average' response of the population as a whole, suggesting that studies of maternal effects which do not incorporate measurements of maternal fitness could lead to incomplete or misleading conclusions. These results provide empirical support for the theory of fitness-associated recombination (Hadany & Beker 2003b; Agrawal et al. 2005), and they suggest that despite the obvious costs for individuals and populations, stressful events such as infections may paradoxically facilitate adaptive evolution (Hoffmann & Hercus 2000; Badyaev 2005; Zhong & Priest 2010).

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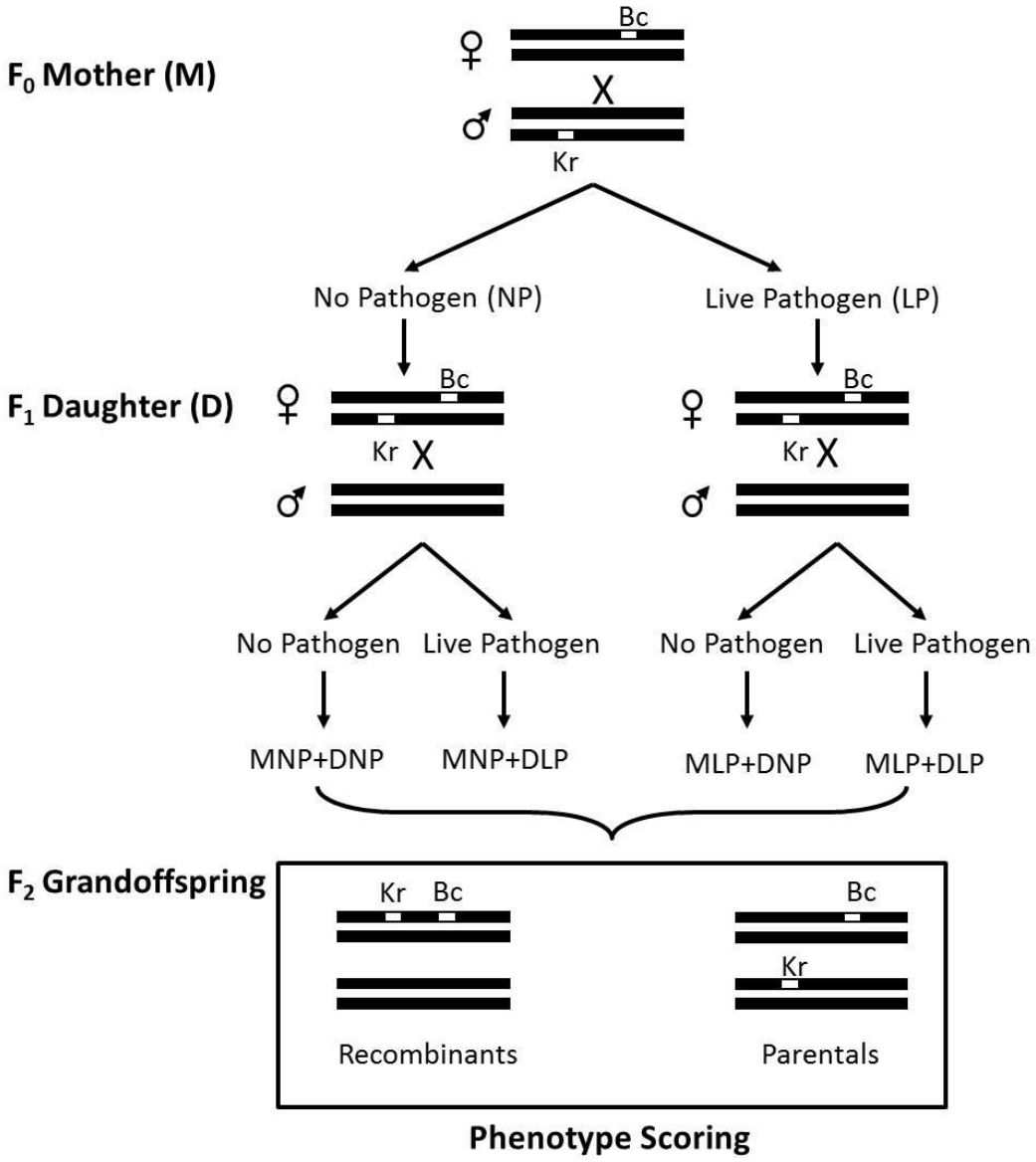

Figure 4.1 Design for the live pathogen experiment. An identical design was used in heat-killed pathogen experiment.

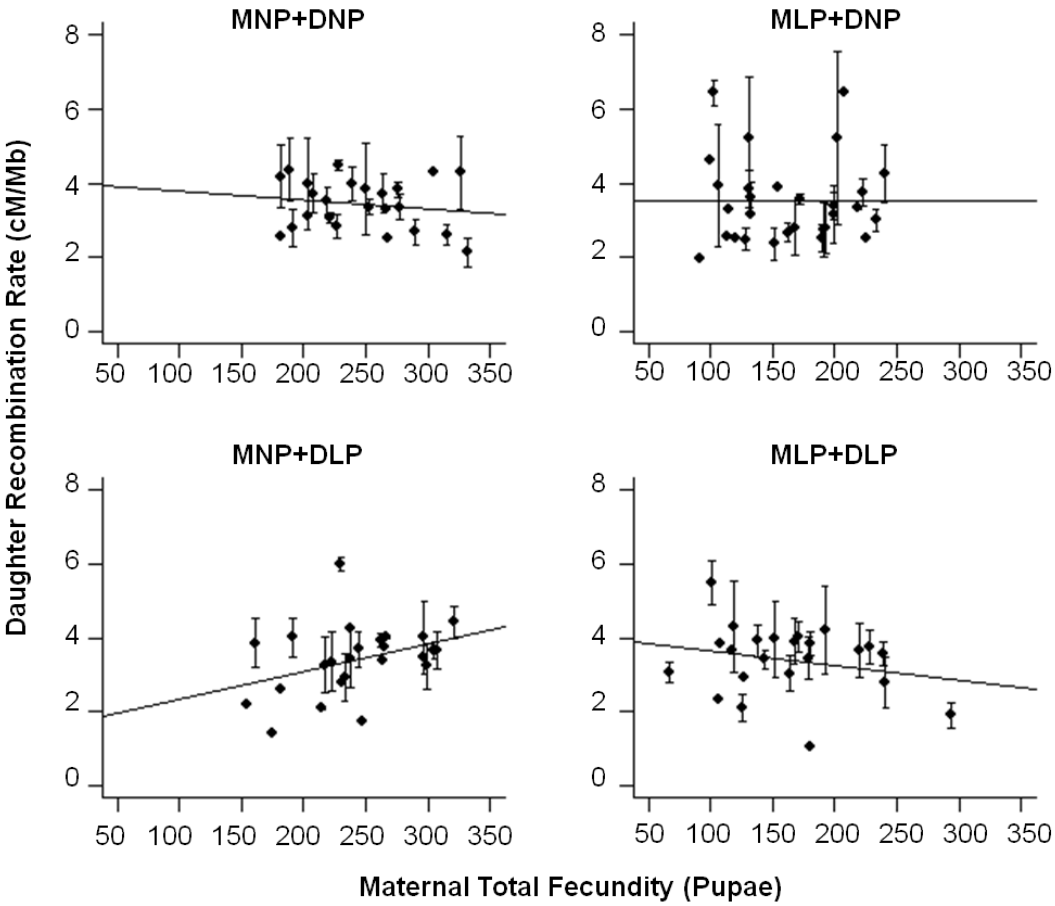

Figure 4.2 Relationships between maternal and daughter meiotic recombination during day 2-4 post-inoculation. Plotted data points are maternal family means with standard errors of the mean. Fitted lines are least squared regressions on maternal family means. Prior to calculating the means, we excluded all daughters for whom fewer than 12 offspring were scored within the 48 hour window. M-Maternal; D-Daughter; NP-No Pathogen; LP-Live Pathogen.

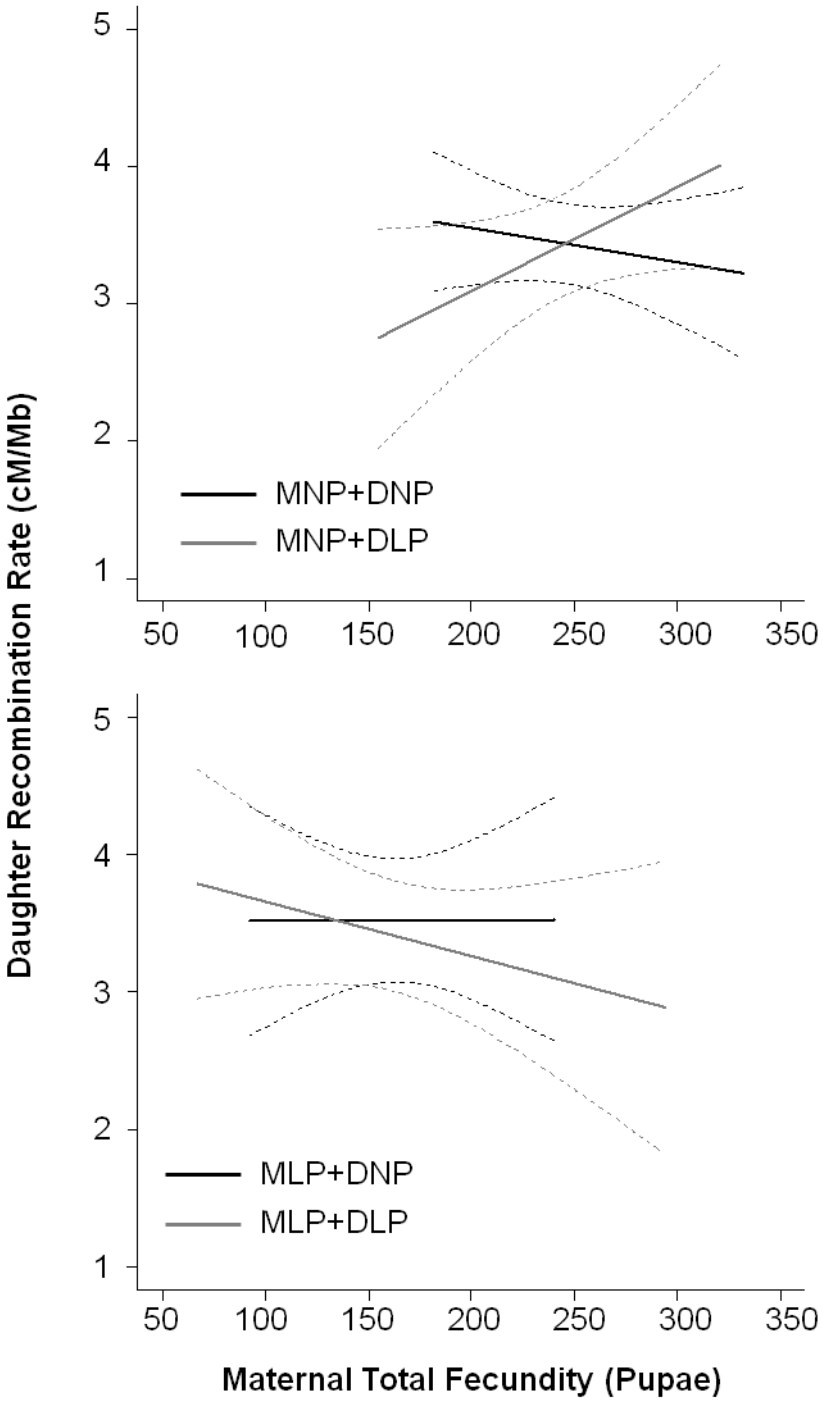

Figure 4.3 Relationships between maternal and daughter meiotic recombination during day 2-4 post-inoculation. Solid lines are least squared regressions on maternal family means. Dotted lines are 95% confidence interval for the regression. Prior to calculating the means, we excluded all daughters for whom fewer than 12 offspring were scored within the 48 hour window. M-Maternal; D-Daughter; NP-No Pathogen; LP-Live Pathogen.

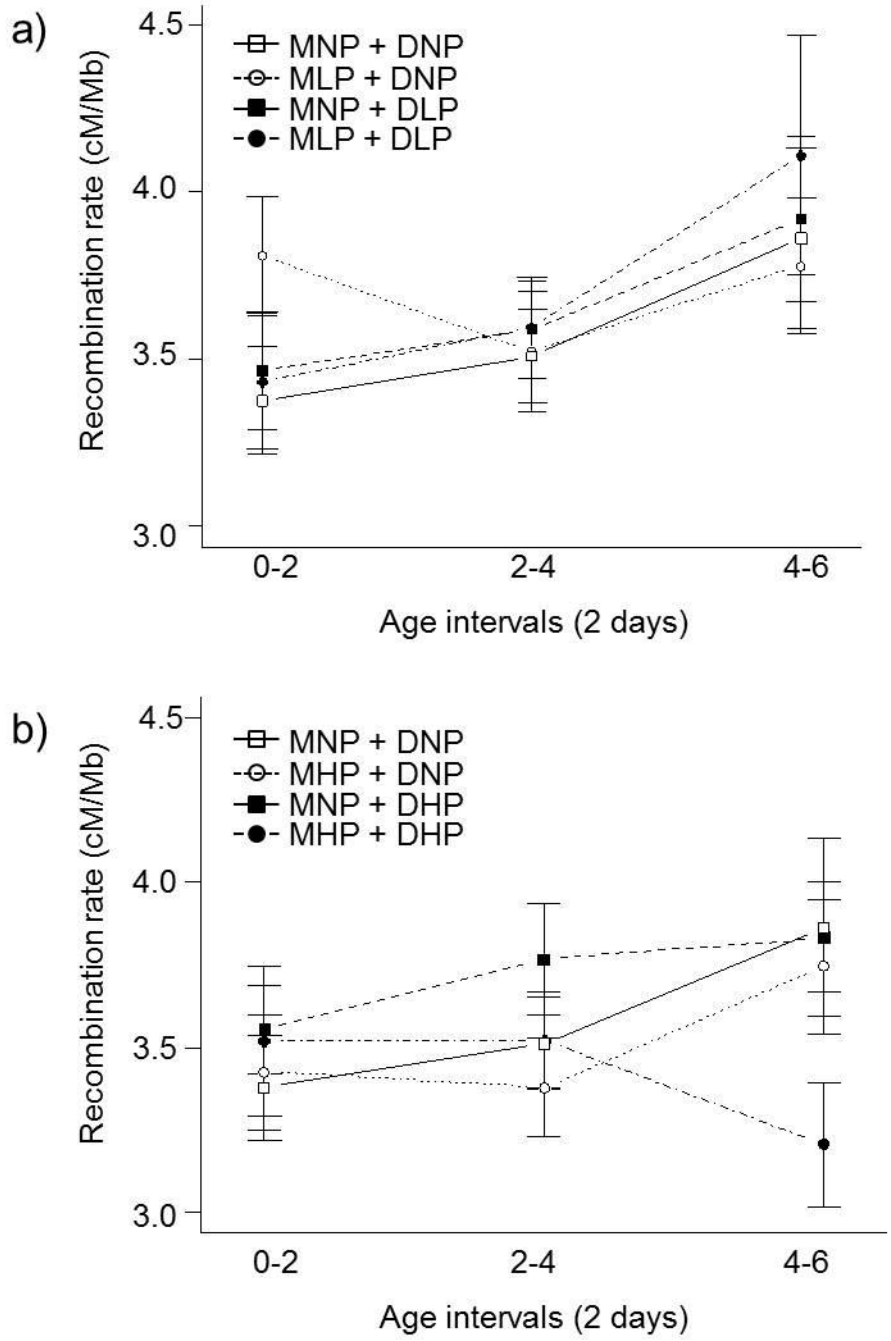

Figure 4.4 Effects of maternal and direct pathogen treatment on daughter age-specific meiotic recombination rate. Displayed data points are mean recombination density for each daughter over a 48 hour window. Prior to calculating the means, we excluded all daughters for whom fewer than 12 offspring were scored within the 48 hour window. Error bars are standard errors of the mean. a) Live pathogen; b) Heat-killed pathogen. M-Maternal; D-Daughter; NP-No Pathogen; LP-Live Pathogen; HP-Heat-killed Pathogen.

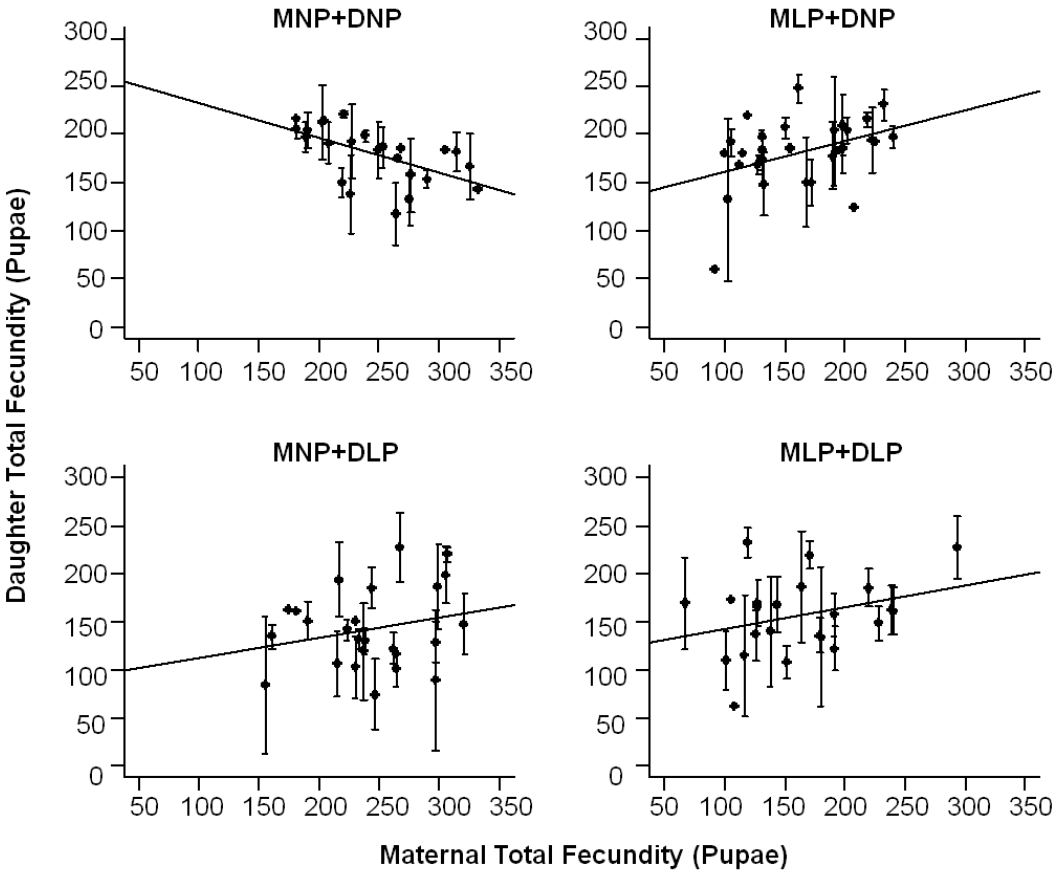

Figure 4.5 Relationships between maternal and daughter total reproductive output across pathogen treatments. Plotted data points are maternal family means with standard errors of the mean. Fitted lines are least squared regressions on maternal family means. M-Maternal; D-Daughter; NP-No Pathogen; LP-Live Pathogen.

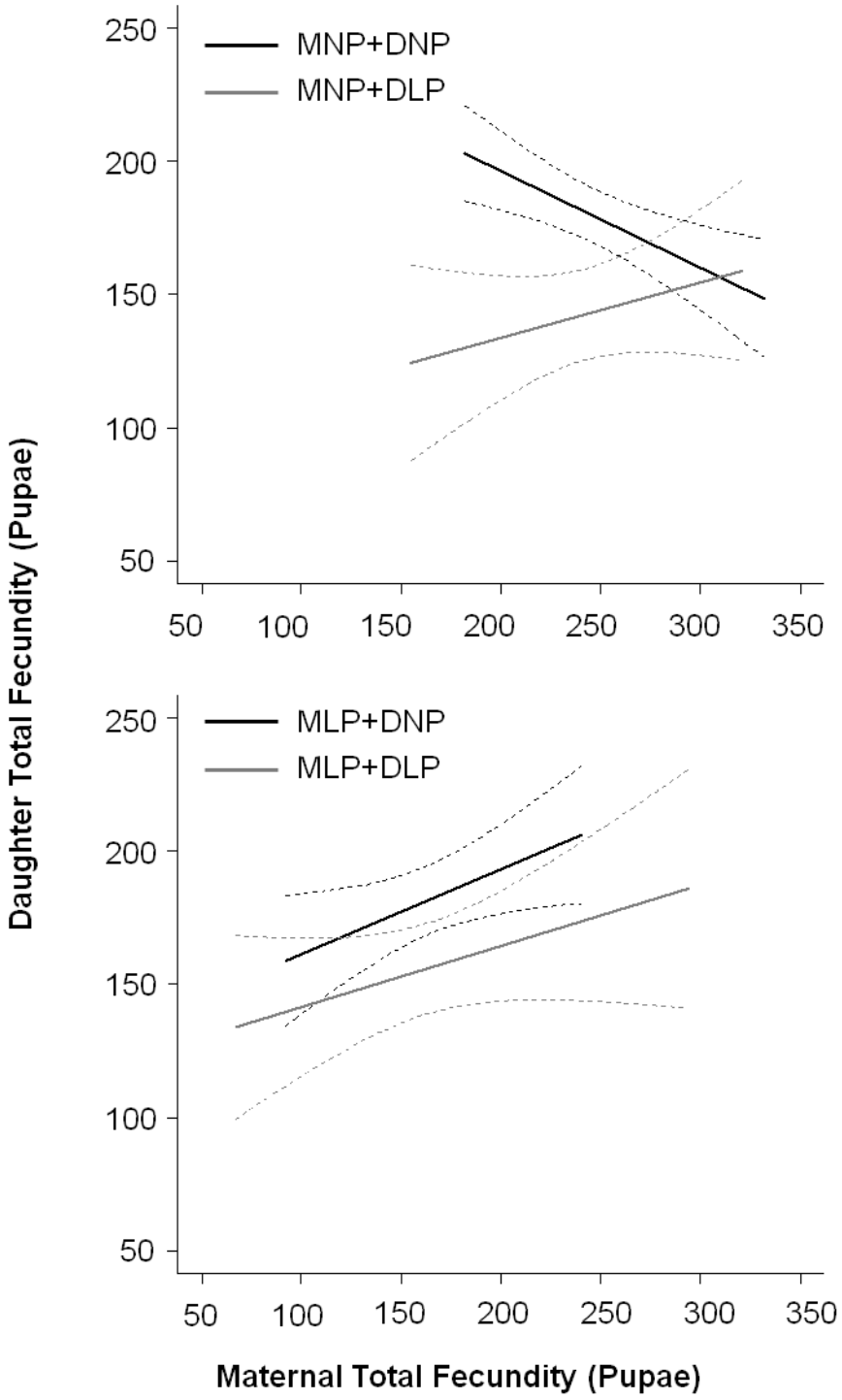

Figure 4.6 Relationships between maternal and daughter total reproductive output across pathogen treatments. Solid lines are least squared regressions on maternal family means. Dotted lines are 95% confidence interval for the regression. M-Maternal; D-Daughter; NP-No Pathogen; LP-Live Pathogen.

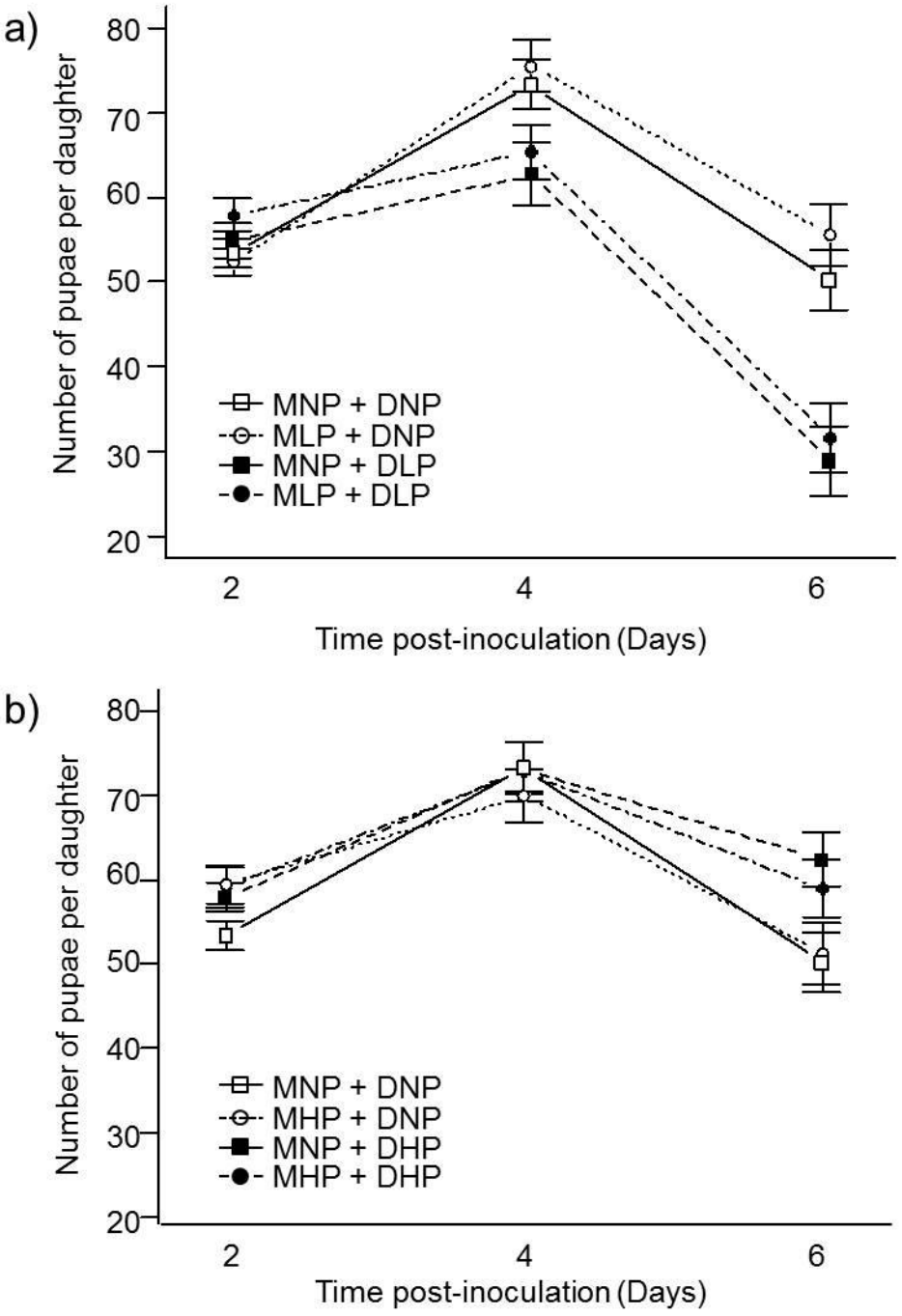

Figure 4.7 Effects of maternal and direct pathogen treatment on daughter age-specific reproduction. Displayed values are number of eclosed pupae for each daughter over a 48 hour window. Error bars are standard errors of the mean. a) Live pathogen; b) Heat-killed pathogen. M-Maternal, D-Daughter, NP-No Pathogen, LP-Live Pathogen, HP-Heat-killed Pathogen.

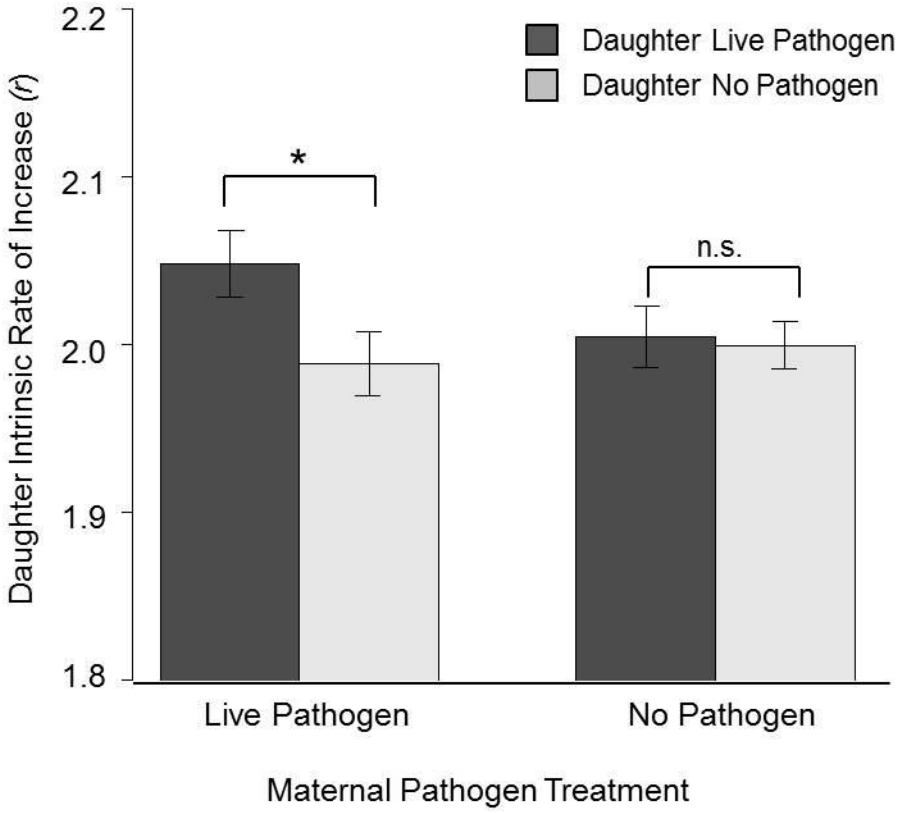

Figure 4.8 Effects of maternal and direct pathogen treatment on daughter intrinsic rate of increase. *r* was calculated for each treatment using blocks as replicates. Error bars are the standard error of the mean. Statistical significance is based on planned contrasts within ANOVA. * indicates $p < 0.05$ and 'n.s' indicates $p > 0.05$.

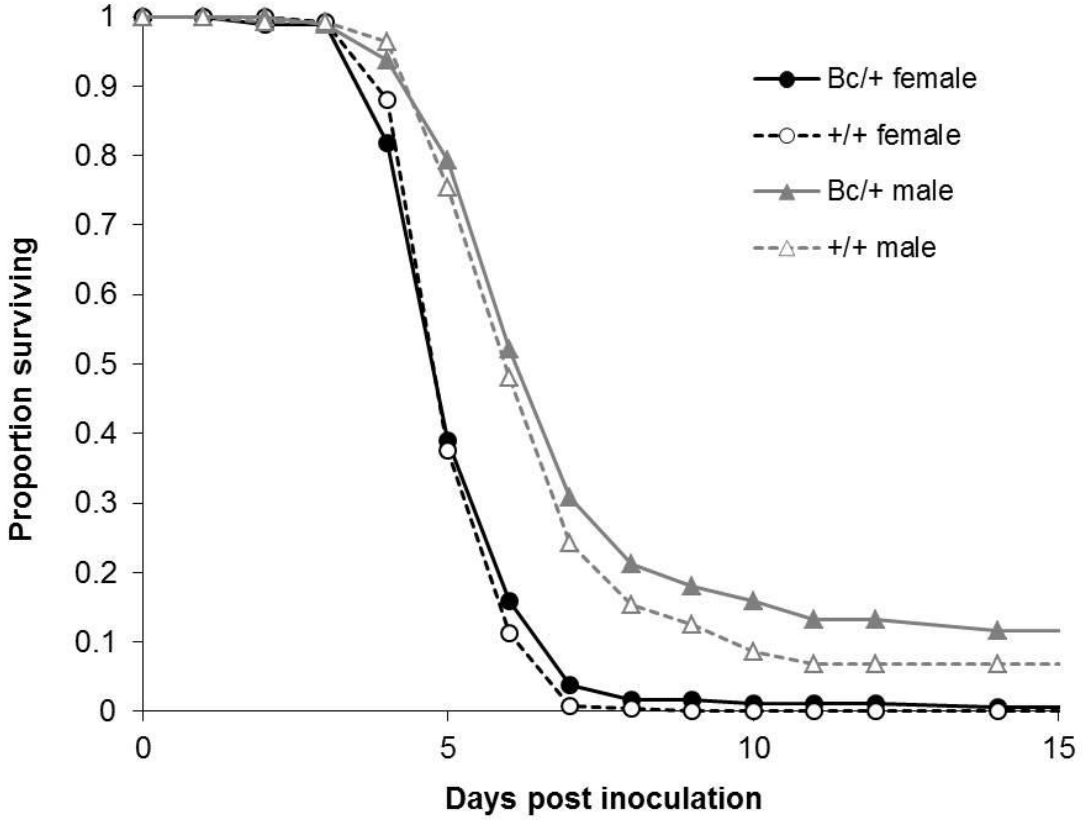

Figure 4.9 The presence of Blackcell mutation does not influence susceptibility of *Drosophila* to *Metarhizium* infection. Adult age 4 day old backcrossed Dahomey wild type (+/+) and black cell heterozygous (Bc/+) flies were infected with 0.05g of conidia and kept in mixed sex cages (n=280 flies with equal sex ratio).

Table 4.1 Generalized Linear mixed effects model estimates of slopes of the relationship between maternal total fecundity and daughter meiotic recombination at day 2-4. Estimates and statistical significance based on separate GLMM models for each treatment including maternal total fecundity with block as a covariate.

Treatment	Effect	SE	Test-statistic (z)	p
MNP+DNP	-0.00136	0.00085	1.60	0.11
MNP+DLP	0.00174	0.00086	2.02	**0.044**
MLP+DNP	-0.00037	0.00105	0.35	0.73
MLP+DLP	-0.00166	0.00079	2.10	**0.036**

Table 4.2 Linear mixed effects model terms and significance for the full model of daughter total fecundity. p values significant at 95% CI or lower are highlighted in bold. Total fecundity in both maternal and daughter generations are the total number of eclosed pupae produced during the first six days post-inoculation.

Model term	**Test statistic and p value**
Maternal Total Fecundity	$F_{1,93} = 0.80$, p = 0.37
Maternal Treatment	$F_{1,93} = 0.81$, p = 0.37
Daughter Treatment	$F_{1,93} = 0.046$, p = 0.83
Block	$F_{11,168} = 3.78$, p < **0.0001**
Maternal Total Fecundity × Maternal Treatment	$F_{1,93} = 0.13$, p = 0.72
Maternal Total Fecundity × Daughter Treatment	$F_{1,93} = 0.89$, p = 0.35
Maternal Treatment × Daughter Treatment	$F_{1,93} = 6.88$, p = **0.010**
Maternal Total Fecundity × Maternal Treatment × Daughter Treatment	$F_{1,93} = 5.99$, p = **0.016**

Table 4.3 Linear mixed effects model estimates of slopes of the relationship between maternal and daughter total fecundity. Estimates and statistical significance based on treatment contrasts within the full model mixed effects model (Table 4.2).

Treatment	**Effect**	**SE**	**Test-statistic (t)**	**p**
MNP+DNP	-0.319	0.148	2.15	**0.034**
MNP+DLP	0.193	0.140	1.38	0.17
MLP+DNP	0.319	0.155	2.06	**0.042**
MLP+DLP	0.122	0.137	0.89	0.37

Chapter 5 General Discussion

Organisms employ a myriad of mechanisms to defend themselves against parasites. There has been tremendous progress in the identification and characterization of the molecular pathways underlying innate immunity (Lemaitre & Hoffmann 2007), but we generally do not understand which of these mechanisms provide effective protection under conditions that are ecologically realistic for the host. In recent years, the field of ecological immunology has emerged as an attempt to understand the diversity and variation in host parasite defence in their ecological context (Sheldon & Verhulst 1996; Rolff & Siva-Jothy 2003). One of the guiding principles of ecological immunology is that immunity comes at a cost, which limits the ability of organisms to simultaneously maintain both high levels of parasite defence and reproductive output. However, although many have argued that we need better estimates of the fitness costs and benefits of immunity (e.g. Graham et al 2010), it is not always clear how this can be accomplished. This thesis is an attempt to better integrate life history theory into the field of ecological immunology. In this final chapter, I will draw together common themes from Chapter 2-4 with respect to the role of life history plasticity and environmental dependence and highlight promising avenues for future research.

5.1 Life history plasticity and parasite defence

The concept of life history trade-off lies at the heart of ecological immunology (Sheldon & Verhulst 1996; Schmid-Hempel 2003). The cost of maintaining immunity on other aspects of life history is illustrated clearly in Chapter 2, where genetic manipulation of the expression of immune genes resulted in correlated changes in survival and fecundity in the absence of infection. In particular, we demonstrated that the expression of *Dif*, a major component of the conserved *Toll* humoral immune defence pathway has substantial costs in terms of both survival and lifetime reproductive success. Though not considered a classic immune gene, we also found that *Turandot M* has an antagonistic pleiotropic influence on the life history of the fly: it enhances fecundity at the cost of decreased survival. The results described in Chapter 3, where the changes in temperature preference of fruit fly exposed to a pathogen adaptively altered their life history, further suggest that host

life history is extremely plastic and tightly integrated with parasite defence; indeed it might be more useful to view patterns of longevity, reproduction and immunity in the context of a broader life history strategy. While many previous studies have described examples of parasite induced changes in thermoregulatory behaviour (e.g. Watson 1993; Adamo 1998; Elliot et al. 2002; Richards-Zawacki 2010), we showed that temperature preference facilitates distinct life history strategies, which are associated with different levels of resistance against fungal pathogens. In particular, our results explain why uninfected hosts should prefer warmer temperature despite the cost to their antifungal immunity. In Chapter 4 we demonstrated that the life history of the host respond to pathogen exposure in ways that extend across multiple generations. Although we did not assess the immunity of daughters directly (also see *5.3.3*), the finding that live pathogen exposure triggered fitness-associated maternal effects on their daughter's life history suggests that mothers can influence the trade-off between reproduction and immunity in their offspring. Finally, by using multiple fitness measures including the intrinsic rate of increase and age-specific reproduction (Chapter 3 and 4), we showed that life history responses to parasites can have dramatically different impact on these measures yielding valuable inferences. However, because of the plasticity of life history, no one fitness measure is likely to be adequate in all situations, which warrants a more pluralistic approach in future studies of eco-immunology.

5.2 Environmental dependence of immunity

There is a growing realization that immune function is highly dependent on the environmental context: experimental results obtained under a single set of environmental conditions are not necessarily generalizable (Lazzaro and Little 2009). In this thesis I have uncovered further empirical support for this general observation and provided evidence that environment-dependent regulation of parasite defence are often adaptive for the host. First, the levels of expression for some immune genes in *Drosophila* appear to be modulated by more external cues than just pathogen-associated molecular patterns described by classic immunology (Chapter 2; Immonen & Ritchie 2012). The pre-emptive upregulation of *Turandot M* in female fruit flies by male courtship song is consistent with immune anticipation of mating (Siva-Jothy 2009), which is expected to be advantageous for the host when mating is

reliably associated with elevated risk of acquiring sexually transmitted infections (Knell & Webberley 2004). Second, we found clear evidence that antifungal resistance in *Drosophila* is dependent on their body temperature, and that infected hosts actively exploit this thermal dependence by moving to colder temperatures (Chapter 3). Together with previous documented examples, our results suggest that parasite-induced thermoregulatory behaviours is a general and important mechanism of immunity in poikilotherms (Thomas & Blanford 2003). Finally, though not typically considered immune response, results reported in Chapter 4 showed that life history and meiotic recombination in one generation can be influenced by the history of pathogen exposure in the previous generation. Such dependence could be beneficial for both the parents and offspring if the parasite environment experienced by the current generation is predictable from maternal parasite environment (e.g. Rice 1983). More generally, the magnitude and direction of maternal effects are dependent on maternal fitness, such that even individuals within the same pathogen treatment responded differently. Thus, experiments that incorporate measurements of individual fitness could reveal further insights into the environmental dependence of immunity.

5.3 Future directions

5.3.1 Methodological advances

Although the *Drosophila-Metarhizium* system has a number of advantages over traditional models favoured in eco-immunological studies (discussed in Chapter 1), the methodology employed in this thesis could be further improved by incorporating a number of new techniques. Previous studies of immunity using entomopathogenic fungi have either placed the insects directly onto sporulating fungal cultures (e.g. Lemaitre et al. 1997; Kraaijeveld & Godfray 2008) or applied fungal suspensions (typically in artificial surfactants) which increased their effectiveness (e.g. Moret & Siva-Jothy 2003). We have used a method that exposed flies to a known weight of freshly collected conidia (also see Taylor & Kimbrell 2007). Although reliable in achieving infection, this method is unsuitable for applying small inoculums. A promising alternative method could be implemented by exposing flies to a treated substrate sprayed with fungal suspension, and for which the density of spores per unit area can be estimated (e.g. Tinsley et al. 2006). This technique would allow flies

to acquire lethal pathogen dose over time through physical contact with the substrate. It could prove particularly useful in administering relatively small doses to the host by restricting contact time (e.g. testing the effect of low-dose fungal infection independent of sexual transmission in Chapter 2).

Future studies of eco-immunology that measure parasite load could benefit from using molecular techniques such as real-time quantitative PCR (RT-qPCR; Bell et al. 2009). Traditional methods for assessing *in vivo* fungal load relies on visual quantification of blastospores in haemolymph or the number of colony forming units (CFUs) of samples grown on fungal culture media. These methods tend to have low sensitivity both when the parasite load is very low (early stage of infection) or very high (typical of late-stage infection). Moreover, they often lack specificity and unable to distinguish multiple co-infecting strains. In contrast, RT-qPCR based methods have the potential to be highly sensitive and specific to the pathogen strain of interest (Bell et al. 2009). Such high quality estimates of parasite load are particularly valuable for making inferences regarding host immune function e.g. tolerance (Chapter 3).

5.3.2 The role of cuticle in insect immunity

Although the role of the innate immune system in resisting fungal infections is well documented, it is only the last line of defence once the barrier defence provided by the cuticle has been breached (Siva-Jothy et al. 2005; Figure 1.1). Because of their unique mode of infection, the cuticle is likely to be a major component of overall defence against entomopathogenic fungi. Insect cuticles contain fungistatic compounds that delay germination of conidia (Gillespie et al. 2000). The procuticle is relatively thick and strengthened by cross-linking proteins via melanisation and sclerotisation. It has been suggested that resistance to entomopathogenic fungi primarily depends on cuticle thickness and the degree of cross-linking (Hajek & St. Leger 1994). This is supported by a recent study in the Greater wax moth, *Galleria mellonella* using the entomopathogenic fungus *Beauveria bassiana* (Dubovskiy et al. 2013a). In general, it appears that selection for increased fungal resistance in *Galleria* is associated with a re-allocation of host defence to the integument, including increased cuticular phenoloxidase activity and stress response (Dubovskiy et al. 2013b). In contrast, humoral immune defences that are activated during the late stage of infection contributes little to antifungal immunity (Dubovskiy et al. 2013a).

Thus, it appears that the critical step in resisting fungal infections in insects is the initial penetration of cuticle. Although genetic variation for antifungal immunity has been demonstrated in *Drosophila* against fungal infections (Tinsley et al. 2006), results from artificial selection experiment for increased resistance has been equivocal (though tolerance might have increased instead of resistance, Kraaijeveld & Godfray 2008). Future studies examining the physiological basis of resistance or tolerance against fungal infections in *Drosophila* and in particular, the role of cuticle, could be very helpful to clarify the role of cuticular defence mechanisms in insects, and the important of barrier defence in general.

5.3.3 Transgenerational immune priming against fungal infections in Drosophila

Parental parasite exposure has recently been shown to improve the immune defence of their offspring across diverse invertebrates (Transgenerational immune priming, TGIP; Little et al. 2003; Moret 2006; Sadd & Paul Schmid-Hempel 2007; Roth et al. 2010). Although we did not specifically test for this, we found no evidence of TGIP in *Drosophila* against *Metarhizium* (Chapter 4). Maternal exposure to live pathogen did not improve the survival of infected daughters by the end of the experiment (surviving/total number of females on day 6-post inoculation: LP+LP (28/63) vs NP+LP (30/72)), which is consistent with a previous study of TGIP in *Drosophila* using bacterial challenge (Linder & Promislow 2009). The apparent absence of immune priming could be due to a number of factors. One trivial explanation is that the large fungal dose used in the experiment overwhelmed any benefit of immune priming. Transfer of maternal immunity to the offspring might also be constrained by its cost to maternal immune function (Zanchi et al. 2012; Moreau et al. 2012). More generally, short-lived species such as *Drosophila* are predicted to invest in inexpensive disease avoidance strategy instead of costly acquired immune 'memory' (Garnier et al. 2012). Nonetheless, within-generation immune priming has been demonstrated in *Drosophila* against both bacterial and fungal pathogens (Pham et al.2007). Thus, future experiments directly testing TGIP in *Drosophila*, perhaps using lower effective pathogen dose, could help to clarify the generality of TGIP in invertebrates.

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Appendix I Stress-Induced Recombination and the Mechanism of Evolvability*

Weihao Zhong and Nicholas K. Priest

Abstract

The concept of evolvability is controversial. To some, it is simply a measure of the standing genetic variation in a population and can be captured by the narrow-sense heritability (h^2). To others, evolvability refers to the capacity to generate heritable phenotypic variation. Many scientists, including Darwin, have argued that environmental variation can generate heritable phenotypic variation. However, their theories have been difficult to test. Recent theory on the evolution of sex and recombination provides a much simpler framework for evaluating evolvability. It shows that modifiers of recombination can increase in prevalence whenever low fitness individuals produce proportionately more recombinant offspring. Because recombination can generate heritable variation, stress-induced recombination might be a plausible mechanism of evolvability if populations exhibit a negative relationship between fitness and recombination. Here we use the fruit fly, *Drosophila melanogaster*, to test for this relationship. We exposed females to mating stress, heat shock or cold shock and measured the temporary changes that occurred in reproductive output and the rate of chromosomal recombination. We found that each stress treatment increased the rate of recombination and that heat shock, but not mating stress or cold shock, generated a negative relationship between reproductive output and recombination rate. The negative relationship was absent in the low-stress controls, which suggests that fitness and recombination may only be associated under stressful conditions. Taken together, these findings suggest that stress-induced recombination might be a mechanism of evolvability.

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Introduction

"The tissues of the body, according to the doctrine of pangenesis, are directly affected by the new conditions, and consequently throw off modified gemmules, which are transmitted with their newly acquired peculiarities to the offspring….
[I]n the cases in which the organisation has been modified by changed conditions…the gemmules cast off from the modified units of the body will be themselves modified, and, when sufficiently multiplied, will be developed into new and changed structures."

Charles Darwin 1868 (pp. 394-397)

As we pass the bicentennial of the birth of Charles Darwin, it seems appropriate to consider not only his ideas that are highly regarded, but also those that are "less fashionable" (Marshall *et al.* 2010, this issue). Darwin's "Provisional Hypothesis of Pangenesis" is a fitting topic in this light (Darwin 1868). Pangenesis was Darwin's solution to the problem of inheritance. It is usually treated as being synonymous with blending inheritance. Though modern genetics has led to its rejection, a key component of the theory may have been overlooked (West-Eberhard 2008). At its core, pangenesis is a theory about evolvability. In modern biology, evolvability has two distinct yet interrelated meanings (Radman *et al.* 1999, Pigliucci 2008; Brookfield 2009). Some define evolvability as a static measure of the capacity of populations to respond to selection, which is captured by narrow-sense heritability or the additive genetic coefficient of variation (Fisher 1930; Houle 1992; for historical reference see Edwards 2010, this issue). Others define evolvability as a dynamic process that generates heritable variation (Wagner and Altenberg 1996; Kirschner and Gerhart 1998). As is evident in pangenesis, Darwin embraced dynamic processes in evolution (Darwin 1868).

In this paper we take a multi-pronged approach to evaluate evolvability as a dynamic process. We discuss historical aspects, with a particular focus on pangenesis. We review the accumulating evidence that the environment has a role in generating heritable variation. We argue that the capacity to evolve might depend on the relationship between physiological and genomic responses to stress. And, we present two empirical studies on stress-induced chromosomal recombination in

Drosophila to illustrate this idea. Our results suggest that the genome might have a dynamic role in facilitating evolutionary change.

In his theory of Pangenesis, Darwin argued that environmental variation could contribute to evolution through the generation of novel heritable variation (Darwin 1868). There are two ways novel environmental conditions and stress can stimulate evolutionary change: by increasing phenotypic variation and/or by increasing genetic variation (reviewed in Hoffmann and Hercus 2000 and Badyaev 2005). The former has been termed phenotypic accommodation (related to the "Baldwin effect", Crispo 2007), which can lead to genetic accommodation, the process by which environmentally induced phenotypic variation becomes constitutively expressed variation after several generations of selection (West-Eberhard 2003). The latter includes adaptive mutation and recombination, which occurs when mutations arise or genetic combinations are generated that allow for rapid adaptation to new conditions. Stress-induced phenotypic and genetic variation can also occur simultaneously. For example, maternal stress can have both genetic and long-term phenotypic consequences for offspring (Badyaev 2005; Priest *et al.* 2007; Priest *et al.* 2008b; Badyaev &Uller 2009).

There are several empirical studies which support genetic accommodation and adaptive mutation and recombination (Waddington 1953; Rutherford and Lindquist 1998; Cairns *et al.* 1988; Fischer and Schmid-Hempel 2005; for reviews see Rosenburg 2001; Parsons 1988 and Badyaev 2005). The number of studies documenting condition specific mutation and recombination is also growing (Agrawal and Wang 2008; Priest *et al.* 2007). Although these studies are compelling, they are not sufficient to deduce whether stress-induced evolutionary change is common or rare. The technical jargon used in the fields of genetic assimilation and adaptive mutation and recombination may also have contributed to the difficulty of testing the general applicability of these theories (de Jong and Crozier 2003, Braendle and Flatt 2006; Moczek AP 2007).

Recent theory on the evolution of recombination (the crossing over of homologous chromosomes during meiosis) provides a much simpler way to evaluate evolvability (Hadany and Beker 2003; Agrawal *et al.* 2005). Recombination has been difficult to explain because its fitness benefits are context-dependent (Feldman 1972; Charlesworth 1976; Barton 1995; Otto and Lenormand 2002). The solution may be that the recombination frequency has evolved to be plastic; frequent in low-

fitness individuals, but infrequent in high-fitness individuals. A series of papers by Lilach Hadany and others on Fitness Associated Recombination show that genes which increase recombination in individuals with low fitness are likely to evolve. If plastic modifiers of recombination exist, then they may contribute to evolvability by increasing additive genetic variation. However, there has been no direct test of the relationship between stress, fitness and recombination (though see Tucíc *et al.* 1981). We know that abiotic and biotic factors affect the rate of recombination (for example, temperature Plough 1917, Grell 1971, Zhuchenko *et al.* 1986; age, Bridges 1929, Redfield 1966; and nutrition, Neel 1941, Davis and Smith 2001). But, the comparisons have usually only been made between treatment and control, not within treatment. In addition, few studies have examined the consequences of acute stress, defined as a brief and sub-lethal exposure to conditions that reduce fitness. Which individuals in a population show most change in recombination rates from bouts of stress? What types of stresses can accelerate evolution? Could stress-induced recombination be a mechanism of evolvability?

Here, we address these questions through two empirical studies of recombination and reproductive output in the fruit fly, *Drosophila melanogaster*. We focused on mating stress, heat shock, and cold shock because they are known to induce stress in fruit flies (Fowler and Partridge 1989; Champan *et al.* 1995; Lindquist 1986: Kelty and Lee 1999; Priest *et al.* 2008a).

Materials and Methods

In Experiment 1, we reanalyzed data from a study on the consequences of mating on the rate of recombination in female fruit flies (Priest *et al.* 2007). Our reanalysis was limited to only one of the three independent marker sets used in the study, Kruppel (Kr) and Black cells (Bc), because it had sufficient linkage distance between markers and large enough sample size to be able to assess the relationship between offspring production and stress-induced recombination. In Experiment 2, we used a longitudinal approach to measure age-specific changes in female offspring production and recombination rate that occurred as a consequence of temperature stress.

Strains and Recombination

We used coupled phenotypic markers to assess recombination frequency in female *D. melanogaster*. For Experiment 1, we obtained lines that expressed 2nd chromosome dominant phenotypic markers, Kruppel (Kr^{If-1}) and Black cell (Bc^{1}), from the *Drosophila* stock center in Bloomington, Indiana. The Dahomey line was generously provided by Prof. Linda Partridge (Partridge and Andrews 1985). We backcrossed each of the markers into the Dahomey line for eight generations to homogenize the background. For Experiment 2, we obtained the Oregon-R line and a line that expressed recessive phenotypic markers for the 2nd chromosome, nub (nub^{1}), black (b^{1}), and purple (pr^{1}), from the *Drosophila* stock center in Bloomington, Indiana. To homogenize the background for the recessive marker lines we backcrossed them into the Oregon-R line for two generations, generated F1s to reconstitute the marker lines and repeated this process two additional times. The tight linkage of both of the marker sets minimised the probability of undetected double recombination events. Each of the marker lines was cultured at 50 eggs/vial for three generations before the start of the experiment to limit environmental variation that arises from differences in larval density. The flies were maintained at 25°C, 50% relative humidity in a 12:12 light cycle.

We focused our analysis on recombination in females because crossing over between paired chromosomes does not occur in male *D. melanogaster*. We assessed the rate of recombination of a female at a particular age interval by determining the proportion of recombinant offspring produced by that female during the interval. In Experiment 1, we scored the proportion of wild type and Bc Kr / + + recombinant offspring produced by Bc + / + Kr heterozygous females. In Experiment 2, we scored the proportion of nub, nub b, b pr, pr, nub pr (double recombinant) and b (double recombinant) offspring produced by nub b pr / + + + heterozygous females that were originally mated with nub b pr / nub b pr males. We focused our assessment of recombination rate on the entire nub-pr interval, rather than for each pair of markers, because double recombinants were extremely rare (4 double recombinants for every 10,000 samples). Estimates of recombination using these methods may underestimate genome wide consequences, because they employ markers in heteromeric regions, while centromeric regions appear to be much more sensitive to environment-induced recombination (Neel 1941).

Stress experiments

The methodological details of Experiment 1 have been previously reported (Priest et al. 2007). Briefly, we collected four-hour-old virgin Bc + / + Kr heterozygous females. When the females were two-days-old they were placed in individual vials with three wild-type (Dahomey) virgin males. At female age 3-day, males were discarded and the females were randomly assigned one of three treatments: High, Medium, or Low mating. Low mating females did not receive any additional exposure to males. The Medium mating females were additionally exposed to three new virgin males for 24 hours at age 5-days. The High mating females were exposed to virgin males every day for eight days. We collected the eggs that were deposited by each female in the bottom of the vials over a 48 hour window, from female age 6-day to 8-day. The females were discarded after their eggs were collected. After the adult offspring had emerged from the vials, they were flash frozen and the phenotypes of all of the offspring were scored. The timing of the mating treatments and egg collection intervals were such that females were prevented from additional exposure to males for 96, 24 or zero hours (Low, Medium and High mating, respectively) before the 48 hour egg collection interval.

In Experiment 2, we collected four-hour-old virgin nub b pr / + + + heterozygous females. At day 2, they were placed in individual vials with three nub b pr tester males. At day 3, the males were discarded. On day 4, 6 and 8, all of the females were transferred to fresh media vials. At day 10 the females were transferred to empty shell vials (with cotton balls pushed close the bottom of the vial to restrict movement) and were randomly assigned one of three treatments: Heat shock, Cold shock, or sham (control) treatment. Heat shock was applied by placing the vials into a 37°C water bath for 20 minutes. Cold shock was applied by putting the females into a freezer that shifted temperature from 18°C to 3°C over a 2.5 hour interval. The sham treatment involved holding the females in shell vials at 25°C for 2.5 hours. After the treatment, the females were placed in media vials, which were randomly distributed and given blind labels. The vials were visually inspected two days later to assess egg production. The females were given an additional day in their vials, to increase the sample of eggs, before they were transferred to fresh media vials on day 13. On day 16, they were discarded. We scored all of the vials, except for a set of day 6 vials that were accidentally washed before they had been scored. The heat shock treatment we used is fairly standard for experiments with live *D. melanogaster*

(Lindquist 1986). Our cold shock treatment involved cooling flies slowly (at 0.1°C/ minute) to induce physiologically relevant cold hardening mechanisms (Kelty and Lee 1999).

Statistical analysis

We calculated a standardized rate of recombination in cM/Mb by dividing each of the recombination values we had measured for each female and age by the estimated physical distance of the marker intervals in mega base pairs. We obtained physical marker distances from FlyBase (Kr-Bc: 7.3 Mb; nub-pr: 7.4 Mb). We calculated 95% confidence intervals for each estimate of female recombination rate using the Wilson score interval (see online supplementary figures). To test for the effects of the treatments on recombination, in both Experiment 1 and 2, we constructed logistic regression models with mating and temperature treatment as fixed effects and the proportion of recombinant offspring as the response variable. We used logistic regression because it weights estimates of recombination according to the sampling intensity. To exclude the possibility that the effects of treatments on recombination rates were driven by a few influential observations or observations derived from females with low fecundity, we repeated the analysis after removing females with low reproductive output. We also repeated the analysis after removing females that were identified as statistically significant outliers using regression deletion diagnostics function "influence.measures" in the *R* statistical software. The results of the logistic regression models without females of low reproductive output or without females that were statistical outliers are qualitatively similar to those using the entire data set (data not shown). In Experiment 2, we also used a repeated measures analysis, which treats female as the unit of replication, to examine the possibility that stress-induced changes in recombination were driven by mortality-induced changes in cohort composition, not physiological changes within females. To test for the effects of the treatments on offspring production, we used analysis of variance (ANOVA), with mating and temperature treatment as fixed effects and the number of offspring as the response variable. For Experiment 1, ANOVA was performed on square transformed data. For Experiment 2, analysis was conducted on untransformed data.

To examine the relationship between recombination and offspring production, we first constructed ANCOVA models which included treatment as a fixed effect, offspring production as a covariate, offspring production × treatment interaction and

the proportion of recombinant offspring as the response variable. We estimated the fitted slopes for each level of treatment in the model to assess the relationship between recombination and offspring production. We used the interaction coefficients of the ANCOVA model to assess differences between the slopes of treatment levels. We repeated this analysis using logistic regression to account for differences in sampling intensity. For Experiment 2, ANCOVA was conducted on natural log transformed data. Each analysis conformed to statistical model assumptions. All statistical analyses were performed using version 2.10.1 of the *R* statistical software (R Development Core Team 2009).

Results

Mating stress

Similar to what was reported previously with the data (Priest *et al.* 2007), there was a significant effect of the mating treatment on the rate of recombination (χ^2= 18.7, df=2,123, p < 0.001; Figure 1). On average, bouts of mating that occurred 0-3 days before the end of the assessment resulted in a 29.3% increase in recombination rate, relative to bouts of mating that occurred 6-days before the end of the assessment (3.22 ± 0.2 cM/Mb for High, 2.62 ± 0.1 cM/Mb for Medium, and 2.49 ± 0.1 cM/Mb for Low mating). There was a significant effect of mating treatment on offspring production ($F_{2,123}$ = 3.06, p = 0.05). High mating treatment females produced more offspring than either Medium or Low mating treatment females, although the only significant difference was between High and Medium mating treatment (Tukey's test: High-Medium, t = 2.42, p = 0.05; High-Low, t = 1.66, p = 0.23; Medium-Low, t = 0.74, p = 0.74).

The effect of mating on the relationship between offspring production and recombination was complex. In the ANCOVA, there was a significant negative relationship (i.e., a negative slope) between offspring production and recombination within the High mating treatment (t=4.3, p< 0.001; Figure 2). This indicates that females with lower offspring production in the two day post-mating egg collection interval have a higher rate of recombination than females that produced many offspring over the same post-mating period. The slopes of the relationship between offspring production and recombination did not differ significantly from zero in the Medium and Low mating treatments (Medium: t = 0.24, p = 0.81; Low: t = 1.58, p =

0.12). Overall, ANCOVA found that the rate of recombination in females was significantly influenced by mating ($F_{2,120}$ = 10.8, p < 0.001), offspring production ($F_{1,120}$ =13.3, p < 0.001) and mating × offspring production interaction ($F_{2,120}$ = 4.0, p = 0.02). However, in the logistic regression analysis, which weights recombination rate estimates according to sample size, the slope of the relationship between offspring production and recombination did not differ significantly from zero for any level of mating treatment (High: z = 0.55, p = 0.46; Medium: z = 0.001, p = 0.97; Low: z =1.99, p = 0.16). Taken all together, logistic regression only found a significant effect of mating (χ^2 = 18.7, df = 2,123, p < 0.001), while offspring production (χ^2 = 1.50, df = 2,122, p = 0.22) and mating × offspring production interaction (χ^2 = 1.04, df = 2,120, p = 0.59) were not significant.

Temperature stress

Before the stress treatment was imposed, there was a decline in the rate of recombination with age in the four egg collection intervals (Figure 3). Significant increases in recombination frequency were detected in the three day interval after the stress treatment was imposed (χ^2 = 49.5, df= 2,23, p < 0.001), but not in the subsequent three day interval (χ^2 = 1.06, df = 2,22, p = 0.59*;* Figure 3). This indicates that the consequences of temperature stress are immediate and short-lived. On average, Heat Shock and Cold Shock increased recombination rate between the nub-pr markers of chromosome 2 by more than ten and five times, respectively, relative to control temperature (Tukey's test: Heat-Control, z = 5.56, p < 0.001; Cold-Control, z = 5.16, p < 0.001; 4.75 ± 1.0 cM/Mb for Heat Shock, 2.67 ± 0.5 cM/Mb for Cold Shock, 0.43 ± 0.2 cM/Mb for Control). There was no significant difference between Heat Shock and Cold Shock treatments (Tukey's test: z = 0.82, p = 0.69; Figure 3). A repeated measures analysis, consisting of the interval before and two intervals after the temperature stress treatment was imposed, revealed a significant positive effect of treatment on recombination (Log Likelihood Ratio test: χ^2 = 13.55, df = 2, p < 0.01). This indicates that the stress-induced changes were driven by changes within females, not mortality-induced changes in cohort composition. There were no significant effects of the temperature stress treatment on offspring production ($F_{2,23}$ = 2.26, p = 0.12).

Similar to the mating treatment in Experiment 1, the temperature stress treatment also altered the relationship between offspring production and

recombination. In the ANCOVA, there was a significant negative relationship between offspring production and recombination within the Heat Shock treatment (t=5.2, p< 0.001), but not within the Cold Shock (t = 0.93, p = 0.36) or Control treatments (t = 0.13, p = 0.90; Figure 4). This indicates the heat stressed females with low fecundity have greater rates of recombination than highly fecund females. The result also shows that heat shock and cold shock have different consequences on the relationship between offspring production and recombination. Overall, ANCOVA found that the rate of recombination was significantly influenced by temperature ($F_{2,20}$ = 39.8, p < 0.001), offspring production ($F_{1,20}$ = 10.5, p = 0.004) and temperature × offspring production interaction ($F_{2,20}$ = 8.8, p = 0.002). The weighted, more conservative, logistic regression analysis revealed similar findings. The slope of the relationship between offspring production and recombination was significantly negative in the Heat Shock treatment (z = 2.36, p = 0.018), and did not differ significantly from zero for either Cold Shock or Control (Cold Shock: z = 0.79, p = 0.43; Control: z = 1.28, p = 0.17). Overall, logistic regression showed that the temperature treatment (χ^2 = 49.5, df= 2,23, p < 0.001) and temperature × offspring production (χ^2 = 6.05, df = 2, p = 0.049) were significant, while the main effect of offspring production was not significant (χ^2 = 2.6, df = 1, p = 0.11).

Discussion

Pangenesis is perhaps Darwin's most puzzling intellectual contribution. In contrast to his other ideas, which were usually well supported by data, pangenesis is more similar to a conjecture than a formal theory. He speculated that organisms could accelerate adaptation to novel environments by secreting cell-specific factors (gemmules) that accumulate in the germline and contribute to heritable trait expression in offspring (Darwin 1868). Although Darwin himself admitted that there was little evidence to support it, he stuck by his theory because he was convinced that it would one day find empirical support: "[My] much despised child, 'pangenesis,' who I think will some day, under some better nurse, turn out a fine stripling (Darwin 1887, p. 120)." Darwin expressed his optimism about the eventual acceptance of pangenesis to many of his colleagues, including Huxley, Hooker, Gray, Hildebrand, Müller, Ogle, Carus and Weir (Stanford 2006). Even when his cousin,

Francis Galton, completed an extensive set of experiments in rabbits that failed to support it, Charles Darwin did not refute pangenesis (Clark 1984).

So, why, in the absence of any concrete empirical support, was Darwin such an advocate for the hypothesis? One explanation is that pangenesis might have reflected Darwin's intuition about the evolutionary process. Darwin's insight was that the material basis of inheritance itself can be open to direct influences of the environment, independent of its effect on mediating natural selection. Though pangenesis had the wrong mechanism of inheritance and incorporated molecular details which, in hindsight, seem fanciful (Charlesworth and Charlesworth 2009), we now know that there are many phenomena that appear to represent cases of evolvability (for example, Waddington 1953; Rutherford and Lindquist 1998; Cairns *et al.* 1998; Hoffmann and Hercus 2000; Rosenburg 2001; West-Eberhard 2003, 2008; Schlichting 2004; Badyaev 2005; Pigliucci *et al.* 2006; Lucht *et al.* 2002; Lui 2008). The problem, however, remains that we do not know whether an organism's capacity to generate heritable variation is a common or rare contributor to phenotypic evolution.

This study was designed to examine the relationships between stress, offspring production and recombination. We did not find the negative between offspring production and recombination in control conditions, which is the pattern predicted by the theory of Fitness Associated Recombination (Hadany and Beker 2003; Agrawal *et al.* 2005). Instead, we found that this relationship is only apparent under particular forms of acute stress.

The consequences of acute stress can be immediate and quite short lived. Previous analysis of Experiment 1 revealed that the bouts of mating only elevate recombination rate for short periods (Priest *et al.* 2007). We found a similar pattern in Experiment 2. Recombination rate was elevated 0-3 days after temperature stress had occurred, but not 3-6 days after it had occurred. Interestingly, though it increased recombination rate, the consequences of cold shock were not associated with offspring production. Taken together, the results indicate that if these conditions frequently occur in nature, then it is possible that heat shock and perhaps also mating stress, but not cold shock, might have a general role in accelerating evolution.

At the outset, the mating stress experiment seemed to be ideal for testing Fitness Associated Recombination because there was a significant effect of the mating treatment on both recombination rate and offspring production. However,

tests of that relationship are complicated by the nature of acute stress. Frequent mating has negative long term consequences for offspring production and survival (Fowler and Partridge 1989; Chapman *et al.*1995; Priest *et al.* 2008a), but acute bouts of mating can also have short term benefits (Wolfner 1997; Priest *et al.* 2008b; Long *et al.* 2010). In Experiment 1, we found that exposure to males actually increased offspring production. Though this did not prevent us from testing for a negative relationship between offspring production and recombination, it may have limited our ability to detect such a pattern. Similarly, though heat stress is thought to have long term fitness costs for fruit flies (Sayeed & Benzer 1996), individual bouts of heat stress generally do not have fitness consequences (Krebs & Loeschcke 1994). In Experiment 2, there was no evidence that heat shock or cold shock affected offspring production. The discordance between acute and chronic stress in both experiments highlights the difficulties in empirically assessing the relationship between fitness and recombination.

Experiment 1 and 2 could have been greatly influenced by a small collection of females with low fecundity, which is a problem because recombination rate estimates are less accurate with small sample sizes (see online supplementary figures). This potential problem was addressed by analyzing the results with logistic regression, which weights observations by sample size. The robustness of the results were further checked by repeating the analysis after removing observations based on small sample size and repeating the analysis after removing observations that were deemed statistically influential. Overall, with only one exception (analyses with logistic regression that involved the High mating treatment), the weighted and non-weighted regressions produced similar findings.

The choice of statistical analysis can influence our ability to detect negative relationships between fitness and recombination. All weighted regression techniques, including logistic regression adopted in this study, necessarily penalize observations that are based on small sample sizes. But, these are precisely the individuals that are expected to be most affected by the treatments if stress-induced recombination is a general mechanism of evolvability. Another statistical issue is that, as we expected, the stress treatments shifted the range of data points in recombination frequency and offspring production. This is problematic because it is inappropriate to test for differences in relationships between treatments when there are non-overlapping data ranges. To compensate for both of these issues, in future work we will need to

increase our sample size and identify ecologically relevant stresses with large effect sizes.

There are many other possible confounding factors that influence our ability to generalize the evolutionary significance of these results. The ones we are most concerned about are body size, genetic variation between *Drosophila* lines, age, culture conditions, larval interactions during development, and even, potentially, the barometric pressure in the laboratory at the time heat stress experiments are conducted (B. Mackowiak and N. K. Priest, personal communication). Though we attempted to distribute some unaccounted for sources of variation through randomization, we simply need more studies of this sort, with substantially larger sample sizes, to be confident of the relationship between stress-induced fitness and heritable variation.

What types of molecules can generate heritable variation?

In his theory of pangenesis, Darwin proposed that an organism's capacity to adapt to novel conditions depends on its ability to produce gemmules, factors secreted by cells that accumulate in the germline and contribute to heritable trait expression in offspring (Darwin 1868). We now know that gemmules do not exist (Charlesworth and Charlesworth 2009). Nevertheless, it is possible that lineages have evolved to express or respond to specific kinds molecules which accelerate adaptation to novel conditions. Though it should only be considered as speculation, it is useful to consider what molecules might have a role in evolvability.

One such candidate molecule is the heat-shock protein, Hsp90, which is thought to capacitate evolutionary change (Rutherford and Lindquist 1998). Other, potential evolvability molecules could be stress hormones or, for that matter, any agent that induces stress or activates a stress-induced cascade. For example, it is well known that bacteria such as *Escherichia coli* can enter a hyper-mutable state via stress-induced genetic pathways in response to stimuli such as nutrient starvation (reviewed in Tenaillon *et al.* 2004).

To identify further candidate molecules, we first need to determine the types of stress organisms typically encounter. J.B.S. Haldane and W. D. Hamilton, in particular, believed that disease has a profound role in evolution (Haldane 1949; Hamilton 2001). Pathogens could be a key source of environmental stress that helps to drive the evolution of recombination (see Fischer and Schmid-Hempel 2005). A

pair of exciting plant studies recently showed that fungal and viral infections can trigger a systemic stress response in the plants, which include defence signal transduction as well as increased recombination in uninfected tissues (Lucht *et al.* 2002; Kovalchuk *et al.* 2003). In addition, since it is quite likely that many small ectotherms such as the fruit fly experience considerable thermal stress upon exposure to direct sunlight (Heinrich 1993), molecules involved in the temperature stress pathway, perhaps even heat shock proteins could also be potential candidates. Lastly, as toxic compounds in male seminal fluid are responsible for mating stress and can stimulate maternal effects which increase the fitness of daughters (Chapman *et al.* 1995; Priest *et al.* 2008b), they may also stimulate recombination (Priest *et al.* 2007).

How can we mathematically characterize evolvability?

One of the reasons the concept of evolvability is controversial is that models of evolvability usually invoke levels of selection above the individual (Pigliucci 2008). Because natural selection lacks foresight and tends to fix alleles that maximise current fitness regardless of the consequences for future evolutionary potential of the population, evolvability is generally not expected to be selected at the level of individuals. The evolution of sexual reproduction and recombination are particularly difficult to explain because neither is likely to provide immediate fitness benefits to the individual expressing it and may even be deleterious for the offspring of the individual if recombination breaks apart existing beneficial allele combinations. Therefore, it is thought that some form of group or even higher levels of selection such as species and clade selection might be necessary for the evolution of evolvability (van Valen 1973; Stanley 1975; Williams 1992; Pigliucci 2008).

In contrast, models of Fitness Associated Recombination (FAR) do not require higher levels of selection. In models of FAR recombination evolves by the spread of modifier alleles which have no effect on the fitness of the individuals that bear them (Hadany and Beker 2003, Agrawal *et al.* 2005). Instead, the modifiers form associations with loci under positive selection and increase in frequencies via hitchhiking on the selective sweeps of beneficial alleles in a population. By demonstrating that rare mutant plastic recombination modifiers can invade populations of uniform recombination modifiers, FAR models show that short-sighted selection at the level of the gene can favour alleles which increase levels of genetic variation and thereby enhance population-level evolvability.

FAR can easily evolve in haploid models (Hadany and Beker 2003), but the relationship is more complex in diploid models (Agrawal *et al.* 2005). Under normal genetic assumptions, plastic modifiers of recombination do not evolve because during meiosis the modifier is just as likely to segregate with low fitness-encoding haplotypes as high fitness-encoding haplotypes (Agrawal *et al.* 2005). However, the modifier may evolve if it is encoded by gene expression in mothers (Agrawal *et al.* 2005). It seems likely that stress-induced recombination could have a central role in models of recombination in fluctuating environments (spatial heterogeneity and Red-Queen dynamic) or if fitness and recombination are also associated with negative fitness interaction between loci e.g. negative epistasis (Otto and Michalakis 1998; Otto and Lenormand 2002; Hadany and Comeron 2008).

There are other possible mechanisms of stress-induced evolvability that need further mathematical treatment. Stress-induced mutation might be a potent force for generating heritable phenotypic variation (Taddei *et al.* 1997; Rosenburg 2001; Agrawal and Wang 2008). Stress might also contribute to evolvability through phenotypic accommodation, the first step in the process of genetic accommodation (West-Eberhard 2003). According to the theory of phenotypic accommodation, previously hidden genetic variation can become expressed after environmental stresses overcome the normally canalized developmental process. If novel phenotypic variation is beneficial in the stressful environment, then selection will favour alleles underlying the selected phenotype. This results in genetic accommodation which stabilizes the expression of the phenotype, i.e. constitutive expression independent of stress exposure (West-Eberhard 2003; Moczek 2007). Though we have emphasized the role of stress, mathematical models of evolvability do not have to invoke stress. Computational models have been used to describe how genetic modularity contributes to evolvability (Wagner and Altenberg 1996). Quantitative genetic models of maternal effects and other indirect genetic effects might also be considered models of evolvability because they allow for the additive genetic variation in one individual to be positively influenced by trait expression in another (Kirkpatrick and Lande 1989; Wolf 2003).

Regardless of the mechanism, it is clear that we will only be able to resolve the general significance of evolvability by testing mathematical models with empirical evidence. This study is, to the best of our knowledge, the first attempt at empirically elucidating the relationships between fitness and stress-induced variation

for ecologically relevant stresses. Our results did not provide evidence for a general relationship between offspring productions and recombination as predicted by FAR. Instead, the expected negative relationship was only found in the heat shock treatment. These results suggest that certain types stress might have the capacity to stimulate evolutionary change. Thus, while future studies utilising larger sample sizes are clearly needed, it is possible that part of the intuition behind Darwin's theory of pangenesis is correct.

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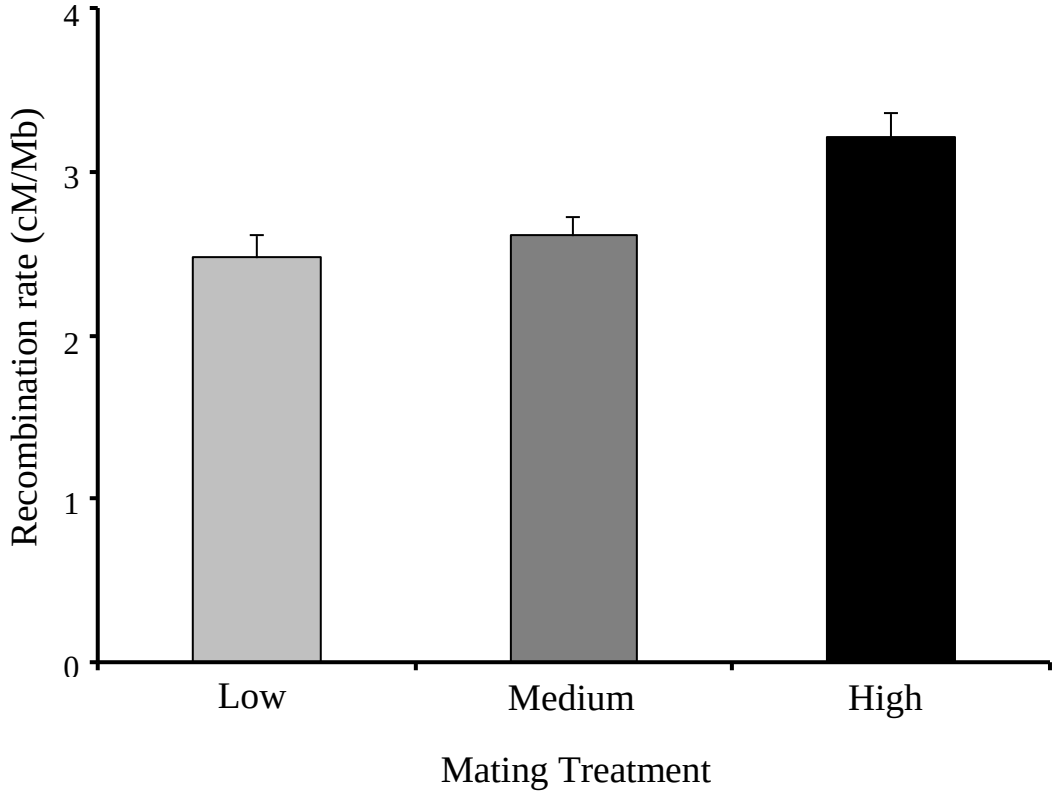

Figure 1. The effect of mating frequency on the average recombination rate in females within the *Kr-Bc* marker interval of chromosome 2 (Data from Priest *et al.* 2007). Sample sizes are displayed above the standard error bars. Mean total offspring production used in calculation of recombination rate were 70.3 (High), 65.2 (Medium) and 62.5 (Low). Differences between treatments were evaluated using Tukey's multiple comparison tests. Significance codes "N.S." ($p>0.5$) and "**" ($p<0.01$).

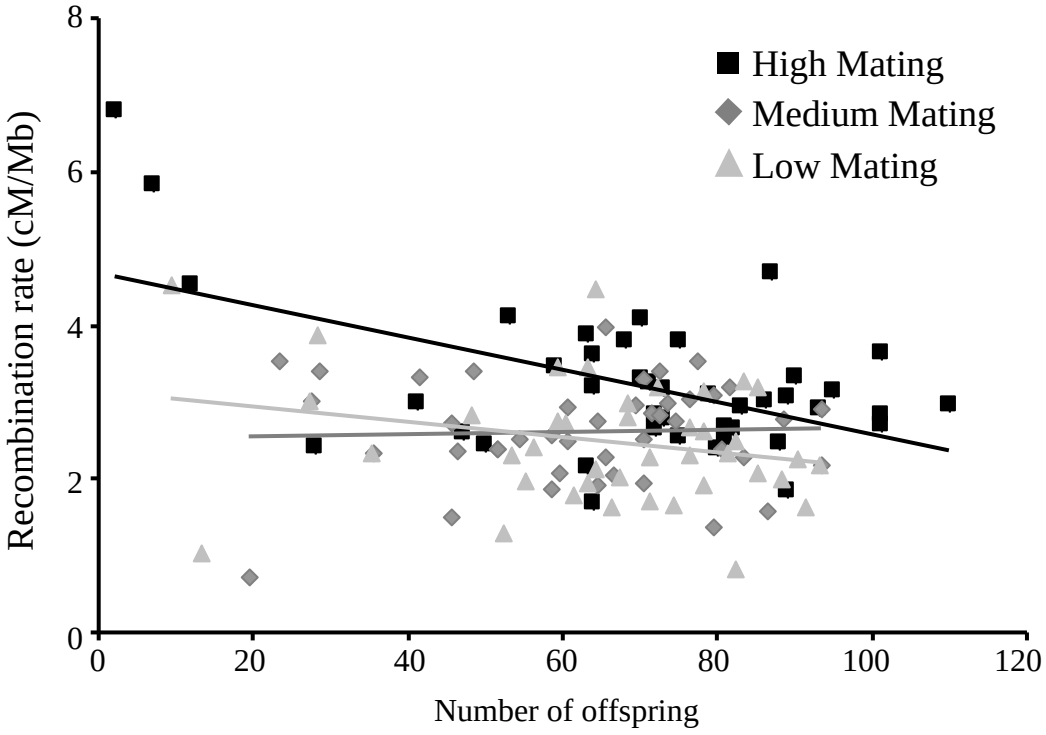

Figure 2. The effect of mating frequency on the relationships between offspring production and recombination rate within the *Kr-Bc* marker interval of chromosome 2 (Data from Priest *et al.* 2007). Each data point represents the estimated value of recombination for a single female over a single three-day (day-13) egg laying period. Sample sizes: 41 (High mating, black square), 43 (Medium mating, grey diamond) and 42 (Low mating, light grey triangle). Fitted lines are linear regressions on untransformed data.

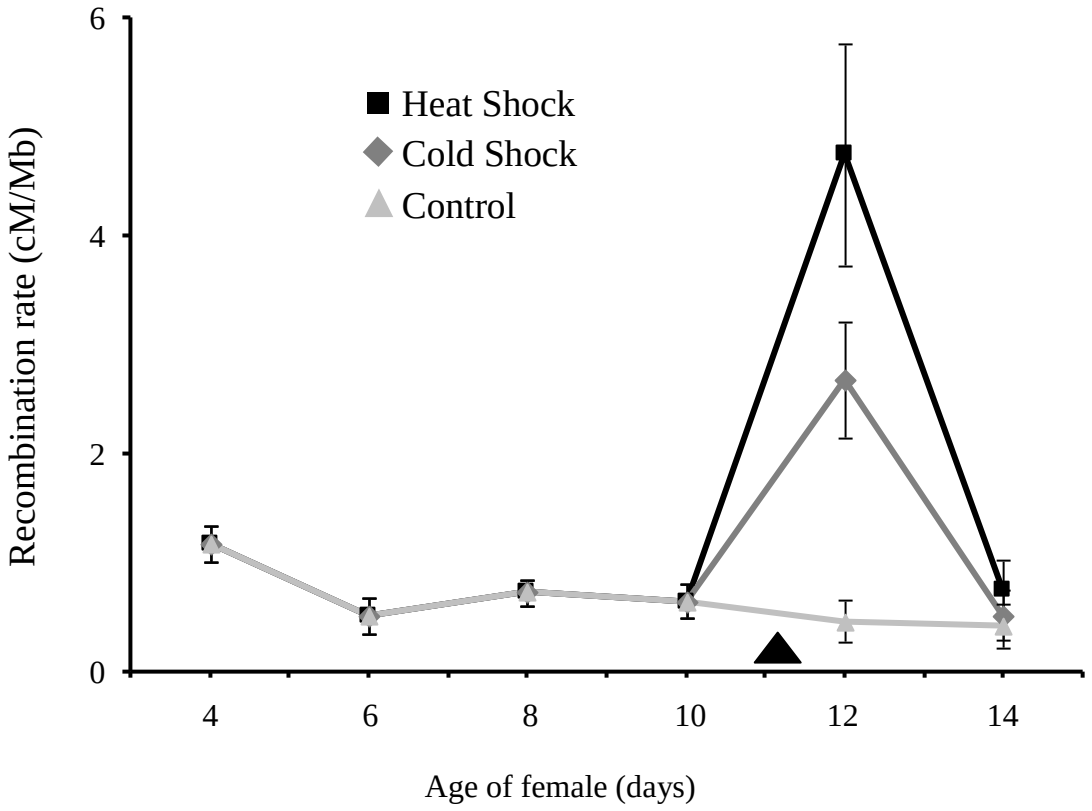

Figure 3. The effect of brief and extreme changes in temperature on average recombination rate in female fruit flies within the nub-pr marker interval of chromosome 2. The flies that survived to day 10 were either exposed to heat shock, cold shock, or sham treatment. The black arrow indicates when the stress treatment was applied. Differences between treatments were evaluated using Tukey's multiple comparison tests on log-transformed data. Significance is indicated by letters above the standard error bars, with different letters indicating a significant differences at p = 0.05.

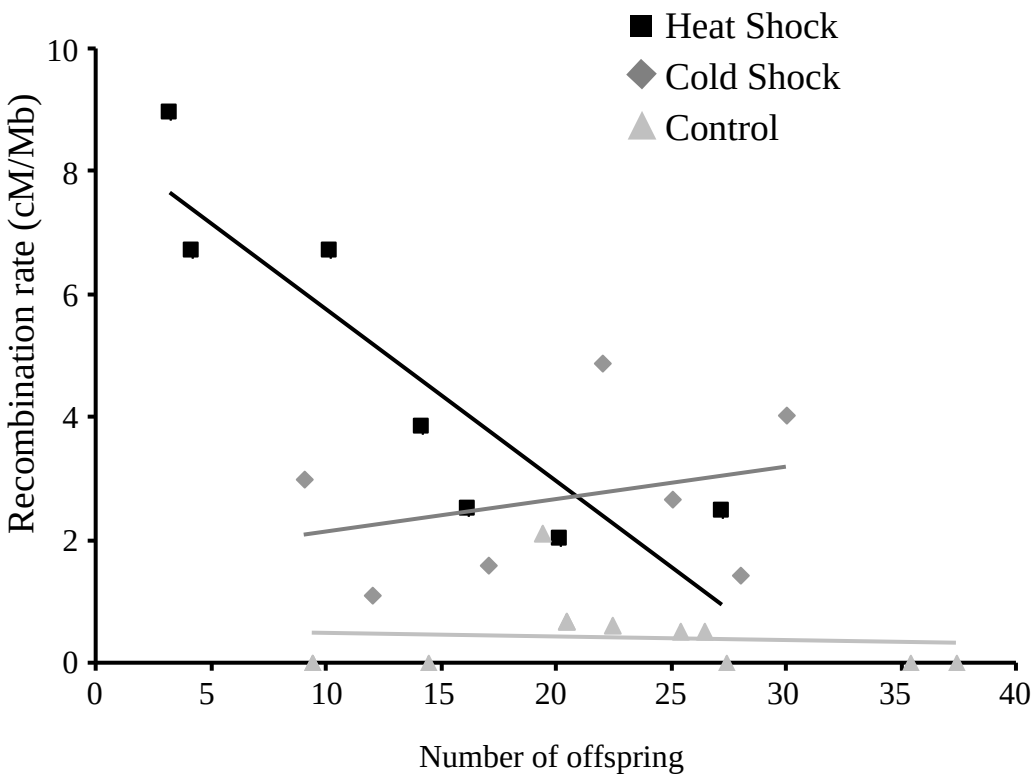

Figure 4. The effect of brief and extreme changes in temperature on the relationships between female recombination rate and female offspring production within the nub-pr marker interval of chromosome 2. Each data point represents the estimated value of recombination for a single female over a single three-day (day-13) egg laying period. Sample sizes: 7 (Heat shock, black square), 7 (Cold shock, grey diamond) and 10 (Control, light grey triangle). Fitted lines are linear regressions on untransformed data.